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SECTION MEETINGS

CLEVELAND, O.	
University Hospitals	November 19, 1956
DISTRICT OF COLUMBIA	
George Washington University	October 4, 1956
ILLINOIS	
Wesley Memorial Hospital, Chicago	November 13, 1956
IOWA	
State University of Iowa	October 30, 1956
MARYLAND	
Baltimore City Hospitals	November 2, 1956
PACIFIC COAST	
University of California, Berkeley	October 24, 1956
SOUTHERN CALIFORNIA	
University of California, Los Angeles	October 18, 1956
SOUTHWESTERN	
University of Texas Dental School	October 19-20, 1956
WESTERN NEW YORK	
University of Buffalo	November 3, 1956

Effect of Environmental Temperature in Traumatic Shock.* (22840)

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The question of effect of environmental temperature upon survival time in traumatic shock has not been definitely answered. Previous workers(1-5) report optimal temperatures ranging from 10° to 35°C. However, this discrepancy is no doubt due to the use of different methods of inducing shock as well as the use of different species of animals. Since control of the environmental temperature in treating human cases of shock is to some extent possible, the question is an important one. This paper is submitted as a contribution toward the study of this problem.

Method. Our method of inducing shock in the rat has been previously described(6). In brief, it consists in withdrawing the cecum

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through a mid-abdominal incision and pinching it at a rate of 3 times per second with a rubber-tipped hemostat for 15 minutes. Nembutal anaesthesia, in a dose of 50 mg/kg is employed and blood pressure recordings from the carotid artery are taken. Data from several hundred control animals used in previous studies have shown this to be a reliable method of inducing irreversible shock. In the present experiment a refrigerator equipped with a heating unit and small fan was used as the constant temperature chamber, the temperature being controllable to less than .5°. The preparatory surgery and infliction of trauma was done at room temperature, which, however, was always within the range of 20° to 25°. The body temperature was taken by means of a dial thermometer, inserted 4 cm within the rat's colon and read at 15 minute intervals. Nine temperatures, between 10° and 40° were studied, 10 rats being used in each group. Males of the University of Denver colony, weighing between 195 and 205 g were employed.

TABLE I. Effect of Environmental Temperature on Survival Time.

Env'l temp., °C	Mean survival time, min.	Stand. dev., min.	Avg body temp. at death, °C
10	63.1	13.0	19.2
15	59.3	12.0	19.2
17.5	127.8	49.2	19.6
20	212.9	57.6	20.2
22.5	171.3	82.8	23.6
25	153.0	22.4	25.5
30	128.8	26.3	30.0
35	91.5	17.4	31.9
40	58.7	16.7	33.6
	20° *vs* 17.5°, t: 3.55, P <.005		
	22.5°, 1.30, not sig.		
	25.0°, 3.06, P <.01		

Results. The results for survival time and average body temperature at the time of death are shown in Table I; Fig. 1 illustrates body temperature behavior for 3 contrasting groups.

Discussion. 1. Survival time. Control groups of 10 non-shocked, anaesthetized rats

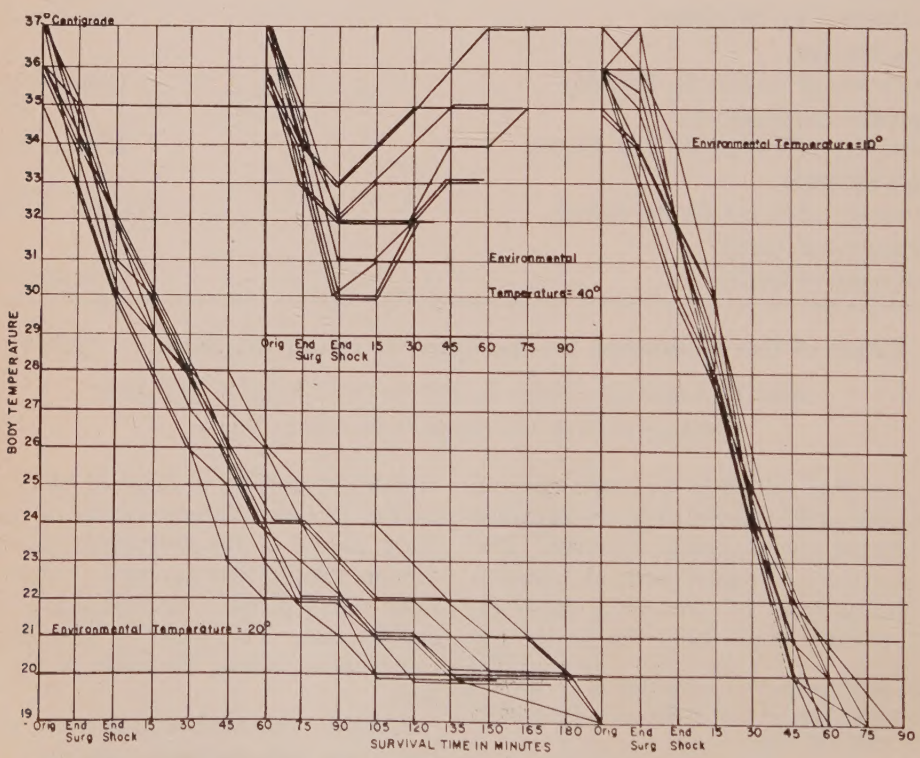

FIG. 1. Trend of body temperature fall of individual rats at environmental temperatures indicated, at 15 min. intervals.

were run at each temperature. At 10°, their mean survival time was 115 minutes, roughly twice that of the experimental group, and at 15°, it was 243 minutes or approximately four times that of the corresponding shocked animals. At all other temperatures, the non-shocked animals were alive and in good condition at the end of 300 minutes. It appears, therefore, that at the 2 lower temperatures, cold contributed directly to the death of the shocked animals, their short survival times not being entirely due to the effect of cold on the shock condition.

It will be seen from the table that the environmental temperature at which survival time was longest was 20° (Mean: 212 min.), although this was not significantly greater than at 22.5° (Mean: 171 min.). It was, however, significantly longer than at 25° (Mean: 153 min.; $p<.01$) and also significantly greater than for the next lower temperature studied, 17.5° (Mean: 127 min.; $p<.005$). Ascending temperatures above 25° caused a progressive reduction of the survival time; at 10° and 15° the survival time was approximately the same (Means: 63 and 59 min.). These results agree with those of Rica *et al.*(3), insofar as 20° being optimal is concerned; we found somewhat lower temperatures less favorable and somewhat higher temperatures more favorable, than did they. The results are also in line with clinical opinion today, which holds that the overheating of shock victims, as practiced in the past, is deleterious. In fact, the difference in survival time at 20° as compared with 30° or more is so great as to suggest the desirability of the use of air-conditioned rooms for shock patients during very hot weather. It will also be noted that the individual variation is greatest at those temperatures where survival time is longest. (S.D. at 20°, 57.6 min. and at 22.5°, 82.8 min.). This suggests that the investigator in the field of shock would perhaps do well to choose a temperature somewhat above or below those figures. The necessity for controlling the temperature, as an important factor affecting survival time, is, of course, obvious.

2. Body Temperature. The average body temperature at the time of death is given in Table I. As shown in Fig. 1 for the 10°, 20° and 40° animals, the body temperature fell by about 5° during the half-hour spent in surgery and traumatization. Upon being placed in the constant temperature chamber, the fall continued precipitately for the low temperature groups, less rapidly for the intermediate temperature groups, and tended to rise for the high temperature group. The control (non-traumatized) groups at 10° and 15° behaved similarly. Their body temperatures at time of death were very nearly the same as those of the experimental group. As previously mentioned, the controls at all other temperatures survived for 300 minutes in good condition. Their body temperatures on termination averaged approximately 10° higher than those of the experimental groups. The results are not strictly comparable, however, since the controls, although given additional anaesthetic when indicated, were never as completely flaccid as were the experimental animals.

It is interesting to note that the survival time of the 2 low temperature groups and the high temperature group is approximately the same, in spite of the great difference in body temperatures, from which it would appear either that both conditions were equally harmful or that the body temperature itself was not the determining factor in causing death.

Summary. The effect of 9 environmental temperatures between 10° and 40° on survival time and on body temperature of rats in traumatic shock was studied. The results indicate: 1. that the optimum temperature is 20°. Survival time at this temperature is significantly better than at 17.5° or 25°; lower or higher temperatures are increasingly harmful. 2. Animals in shock appear to be poikilothermic, their body temperatures tending to approach the environmental temperature.

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Isolation of Intranuclear Inclusion Producing Agents from Infants with Illnesses Resembling Cytomegalic Inclusion Disease.*† (22841)

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On morphologic grounds various authors have suggested that the syndrome termed "generalized cytomegalic inclusion disease" by Wyatt *et al.*(1) or simply "inclusion disease" by Smith and Vellios(2) is viral in etiology and is a generalized form of salivary gland inclusion disease. The demonstration by Fetterman(3) that the infant with inclusion disease may excrete inclusion-bearing cells in the urine has resulted in evidence suggesting that the generalized process may be more common than hitherto suspected and may not have a fatal outcome(4). Information as to the nature of the causative agent is now being elaborated. Smith investigated the salivary gland viruses of rodents(5). From mouse salivary gland tissue an intranuclear inclusion producing virus was isolated in cultures of mouse tissues. Then, employing cultures of human myometrial cells, Smith obtained a cytopathic virus from human salivary gland material and recently isolated a similar agent at autopsy from kidney tissues of an infant with inclusion disease(6). Related viruses also have been recovered from adenoid tissues of children by Rowe and his coworkers(7). Our interest in the problem arose from the concurrent isolation of an agent from human liver biopsy material as has been noted briefly(7,8).

In this communication we are reporting the isolation of cytopathic intranuclear inclusion producing agents from 3 infants during life; each was ill with a syndrome resembling cytomegalic inclusion disease. One virus, derived from liver biopsy material, has been maintained for 20 months in serial passage. From the second child virus was obtained on 3 occasions from the urine, and once from liver biopsy material. Recently, an agent has been isolated from the urine of a third patient.

Materials and methods. Tissue cultures: Roller tube cultures of various human and animal tissues were utilized. The nutrient fluid at first consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics, soybean trypsin-inhibitor (omitted in kidney cell cultures), and phenol red as previously employed(9); for the past 12 months Hanks'(10) balanced salt solution (45%) has been included and the amniotic fluid reduced proportionately. Changes of nutrient fluid were made at 3- to 5-day intervals. For histologic studies, tissues were planted on coverslips or prepared by the collodion technic of Cheatham(11); fixation was with Zenker-acetic or Bouin's and preparations were stained with hematoxylin and eosin. For serial cultivation, inocula consisting of 0.2 ml of coarsely ground tissue removed from the preceding set of cultures were employed. Control cultures were similarly inoculated and maintained in par-

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¶ Post-doctoral fellow, National Foundation for Infantile Paralysis.

allel. Titrations of viral content of fluids from infected cultures were performed on materials centrifuged at 3000 rpm for 15′; 10-fold dilutions in medium were prepared and groups of 4 cultures of foreskin inoculated with 0.2 ml amounts of the supernate. Such cultures were observed for a minimum of 28 days. The serologic technics employed are described below.

Cases studied and handling of specimens. Case #1: K.D. entered Children's Medical Center on 1/11/55 at age of 3 months for investigation of microcephaly. Examination revealed: (1) jaundice, (2) hepatosplenomegaly, (3) periventricular cerebral calcification, (4) bilateral optic atrophy and one area of chorioretinitis. Urine contained a few white cells; no inclusion bodies could be demonstrated. On 1/20/55 a liver biopsy was performed. Congenital toxoplasmosis was considered in the differential diagnosis and through the courtesy of Dr. W. D. Winter, Jr. a portion of the biopsy as a 20% suspension in physiologic saline was furnished for possible isolation of *Toxoplasma in vitro*. This was inoculated in 0.2 ml amounts into cultures of human embryonic skin-muscle tissue and a cytopathic agent (Davis strain) recovered. Histopathological examination of liver biopsy showed diffuse alteration of architecture with extensive bile stasis. Many degenerate hepatic cells and scattered multinucleate giant cells were present. There were focal areas of erythropoiesis and numerous mononuclear inflammatory cells in the portal areas. Later, additional sections were examined and rare large cells of cytomegalic type with intranuclear inclusions were found. Through the courtesy of Dr. Carl M. Haas, K.D. was seen on 8/9/55; the patient was well nourished but had hepatosplenomegaly; there was clinical evidence of cerebral damage. *Case #2:* B.K. was born 5/3/56. Petechiae, jaundice, and hepatosplenomegaly were noted 8 hours after birth. Roentgenologic studies showed no abnormalities, and there was no evidence of Rh or major blood group incompatibility. In the stained sediment of urine collected on 5th and 8th days of life, large epithelial cells were observed that contained multiple round homogeneous brightly eosinophilic cytoplasmic inclusions; no intranuclear inclusions were seen. Through the courtesy of the Pediatric Service, U.S. Naval Hospital, Chelsea, and in particular of Drs. R. S. Wicksman, T. Delaney, and R. Kluge, materials were collected for study. On 14th day of life urine, spinal fluid, and blood were obtained, transported in ice bath, and inoculated into tissue cultures within 2 hours of collection. The urine was centrifuged 20 min. at 2500 rpm (Internat. #2; head 240), and supernatant inoculated in 0.1 ml to 1 ml amounts into cultures of human foreskin and of human kidney cells. The blood clot was ground with equivalent volume of culture medium and similarly inoculated in 0.4 ml quantities, and the spinal fluid was used as received. Only the foreskin cultures inoculated with urine developed specific cytopathic changes (Kerr agent). On 25th day of life a liver biopsy was performed, and a 10% suspension of tissue in medium prepared; 2 foreskin cultures were inoculated and these subsequently developed focal degeneration. Histologic examination of the biopsy showed preservation of lobular pattern, but loss of cell cord pattern with formation of many large multinucleate giant cells. Rare foci of liver cell necrobiosis were present. A diffuse inflammatory reaction consisting of mononuclear cells, eosinophils, and polymorphonuclear cells was present throughout the parenchyma and in the portal triads. Bile stasis was present within liver cells. No cells with intranuclear inclusions were seen. The picture was identical with that of "neonatal hepatitis" described by Craig and Landing (12). Two foreskin cultures inoculated with urine collected on 36th day of life developed characteristic cytopathic changes. At age of 2 months, B.K. appeared normal except for persistence of hepatosplenomegaly. Yet, virus was again recovered from urine collected on 91st day of life; each of 4 cultures inoculated with 0.1-0.2 ml of urine supernatant showed characteristic focal changes. *Case* #3: (History and materials supplied through kindness of Drs. Hattie E. Alexander and Katherine Sprunt, Babies Hospital, New York.) E. Esp., born 5/9/56, had hepatosplenomegaly and periventricular cerebral

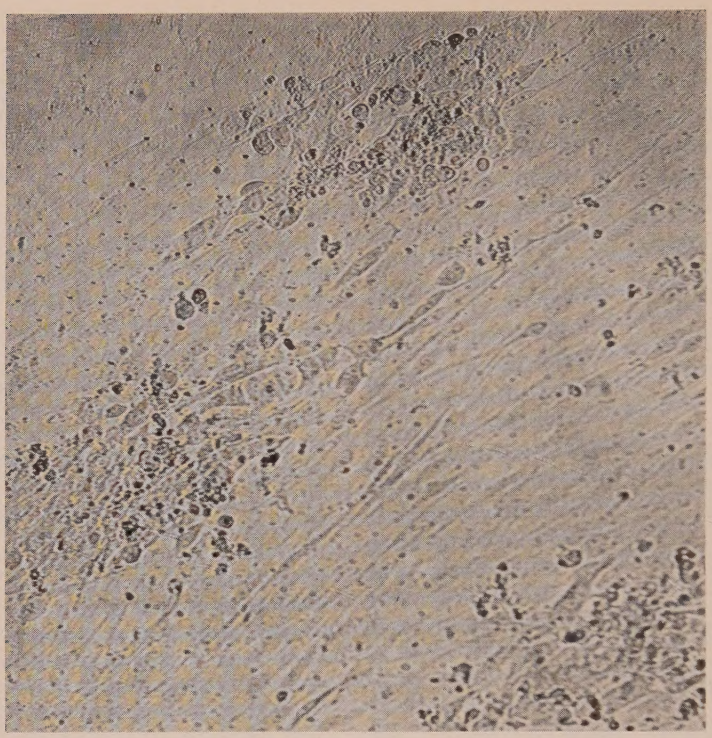

FIG. 1. Culture of human embryonic skin-muscle tissue 34 days after inoculation with Davis liver biopsy material showing 2 focal lesions with prominent pigment granules. × 135.

calcification by roentgenologic examination at 6 weeks of age. Subsequently, progressive chorioretinitis developed. Cytomegalic inclusion cells were demonstrated in the urine on several occasions. On 8/13/56, urine forwarded in melting ice was inoculated into 6 foreskin cultures; specific changes developed in all (Esp. strain).

Results. a. *Isolation of cytopathic agents.* (1) *Davis strain from liver biopsy material*:‡ On 12th day after inoculation of cultures of human embryonic skin-muscle tissue, focal collections of 2-10 swollen, rounded, translucent cells were observed in sheets of fibroblastic outgrowth. In 48 hours the foci had increased in size and a few rounded cells had become irregularly oval or leaf-shaped; the latter were noted to contain refractile, greenish-brown granules that varied considerably in size. Certain of the involved cells also contained masses of smaller, more regular, and less distinct non-pigmented granules. At this stage, degeneration was not a prominent feature, although rare large cells appeared to be undergoing dissolution, releasing granular debris. In one culture progression of cytopathic changes was observed for additional 30 days. The number of foci slowly increased until there were 1 or 2 per low power field. The individual focus, however, increased slowly in size, and tended to remain lenticular in outline. The central area of each often contained brownish granular debris as well as scattered swollen cells (Fig. 1). Two control cultures showed no unusual changes.

(2) *Kerr strains from urine and from liver*

‡ *Special groups for serologic studies*: The circumstances of isolation of the Davis strain suggested investigation of children with toxoplasma-like syndromes but with negative Sabin-Feldman dye test findings. Specimens of serum from patients with these criteria were kindly supplied by Dr. H. A. Feldman. Selected patients, with mental deficiency, have been studied at the Wrentham State School through the courtesy of Drs. K. V. Quinn and D. H. Jolly. We are indebted to various physicians for supplying us with specimens of serum, especially Drs. A. M. Margileth, G. H. Fetterman, A. E. McElfresh, M. Birdsong, R. B. Lawson, and S. W. Wright.

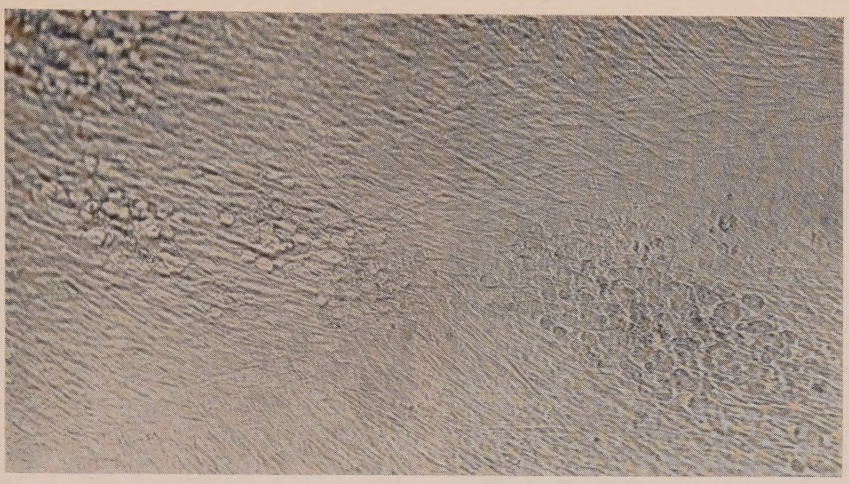

FIG. 2. Culture of human foreskin 20 days after inoculation with urine from baby Kerr showing early focal lesions. × 130.

biopsy material: Focal lesions were first observed in foreskin cultures inoculated with various urine specimens from B.K. after 12, 6, and 9 days respectively, and on 10th day in cultures receiving liver inoculum. Appearance and type of progression of cytopathic changes closely resembled that seen in cultures inoculated with the Davis liver material. (Fig. 2). Again, control cultures failed to develop similar changes.

(3) *Esp. strain from urine*: In the 6 cultures of foreskin tissue inoculated with urine, rounding of scattered fibroblasts was first noted on 5th day after inoculation. From such cells slowly progressive focal lesions developed that contained much dark granular pigment and were similar to those induced by the Davis agent.

b. *Serial propagation of agents*: Serial propagation of the Davis strain was accomplished in cultures of foreskin or of human embryonic skin-muscle tissue employing inocula consisting of ground infected tissue material. Two lines of the Davis agent were established from original cultures. "A" line was passed at intervals varying from 5 to 101 days and has been maintained in culture for 20 passages for 494 days. The "B" line has been maintained in culture continuously 544 days, during which 15 passages were made; in this instance subculture was performed at intervals varying from 8 to 113 days. In both lines, evidence of adaptation to *in vitro* conditions was apparent. Virus was not demonstrable in fluid removed from the original cultures. In the 3rd passage (cumulate period 134 days in culture), virus was present in low titer in the centrifuged fluid as assayed in foreskin cultures. The titer of virus in fluid removed on 25th day after inoculation from the 10th passage (314 cumulate days) was $10^{-2.5}$ and in that removed on 14th day from 13th passage (392 cumulate days) was $10^{-4.6}$. Focal lesions appeared in the original subculture tubes after intervals ranging from 7 to 18 days and persisted as such. In subsequent passages the initial focal lesions were usually followed by generalization of the cytopathogenic process after a varying period of incubation. In the later passages, when large inocula were used, the prepatent period shortened markedly, and the initial cytopathic changes often were generalized rather than focal. For example, in the 17th passage, a diffuse rounding of fibroblasts was noted 24 hours after inoculation. Yet, in this and subsequent passages when inocula approaching the limiting dilution were employed, initial lesions characteristically were of a focal nature and only appeared after a 15- to 28-day latent period. Such focal lesions were, however, usually followed after a few days of incubation, by a generalized involvement of susceptible cells. Two strains recovered from urine of B.K. have been propagated for cumulate periods to 103 days and

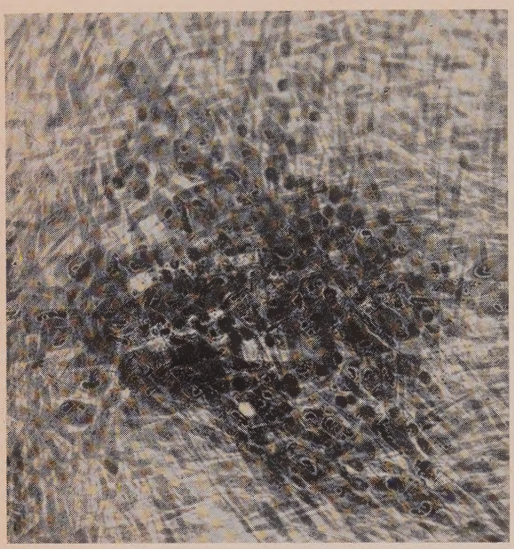

FIG. 3. Stained preparation from foreskin culture 20 days after infection with Davis agent (3rd T.C. pass.) showing discrete focus surrounded by unaltered fibroblasts. (H + E × 145.)

for 2 and 3 subcultures. The focal cytopathic changes resemble those of the early passages of the Davis agent. The Esp. strain has been subcultured once with similar results.

Through the courtesy of Dr. Rowe, the Ad. 169 virus recovered from pharyngeal tonsillar tissue(7) was furnished as 1st passage material. This agent was propagated in cultures of foreskin tissue for 8 passages during 435 days. The morphological changes were similar to those observed with the Davis strain.

c. *Some biologic properties of the agents*: (1) *Cytopathic changes in stained preparations*: Examination of stained materials from foreskin cultures infected with Davis virus revealed a marked alteration of nuclear structures and also changes in cytoplasm of infected cells (Fig. 3 and 4). The earliest nuclear changes noted consisted of the presence in the nucleus of 1 to 4 minute granular bodies that stained amphophilic or weakly eosinophilic. In nuclei in which slightly larger inclusions were present, there was loosening and coarsening of the chromatin reticulum with apparent coalescence of fragments thereof with nucleolar remnants that persisted as one or more prominent bodies near the nuclear membrane. The developing intranuclear inclusions often showed struc-

FIG. 4. Stained preparation from foreskin culture 20 days after infection with Davis agent (3rd T.C. pass.); edge of focal lesion with central degeneration and infected cells at periphery (H + E × 300).

tural differentiation with a narrow, deeper staining marginal zone and occasionally a discrete central body. Some of the larger inclusions appeared to be composed of masses of small particles of this nature. At maximum size the swollen nucleus was occupied by 1 or more large granular, moderately eosinophilic inclusions separated from the marginated nuclear chromatin by a clear zone. In degenerating cells, the inclusion occasionally resembled a honeycomb-like reticulum. Cytoplasmic changes were noted in involved cells in the focal lesions, but were more obvious in preparations wherein generalized spread had occurred. Examination of material from higher passages shortly after inoculation indicated that early rounding of fibroblasts was accompanied by cytoplasmic changes that preceded the appearance of intranuclear bodies. In a paranuclear position, and frequently lying in an indentation of the nucleus, there usually was an oval zone where the cytoplasm stained lighter, more eosinophilic, and appeared to be composed of a mass of fine granules. Infected cells apparently about to undergo dissolution often were noted to contain one to several rather large, round, hyaline amphophilic to eosinophilic cytoplasmic inclusions; in areas of cell destruction these bodies could be seen free in the cellular debris. Stained preparations from cultures infected with the Kerr and Esp. strains showed morphologic changes indistinguishable from those observed with the Davis virus.

(2) *Cytopathic range. Human tissues*: After *in vitro* infection with the Davis virus, focal or generalized cytopathic changes developed in fibroblasts derived from human foreskin, from human embryonic skin-muscle or lung tissue, and in a cultured line of fibroblasts obtained from Microbiological Associates. Similar changes occurred in cultures of human myometrial cells and in the outgrowth from human testicular tissue. The relative sensitivity of certain of these tissues as indicators of virus is suggested by the results of titrations summarized in Table I. The inoculation of cultures of human epithelial cells of various types was not immediately followed by the development of overt cytopathic changes. However, in stained preparations, changes could be demonstrated. In foreskin, the marginal cells in plaques of squamous epithelium in contact with fibroblastic foci often showed changes with intranuclear inclusions; destruction of involved squamous cells occurred at a comparatively slow rate. Variable results were obtained in cultures prepared from trypsinized human kidney cells. In one experiment, where the use of a ground tissue inoculum permitted implantation of infected cells, stained preparations showed limited involvement of cells that probably were of renal epithelial origin. More convincing evidence was afforded by an experiment inoculated with centrifuged infected fluids (Davis, 8th passage) in which staining at the 35th day of incubation revealed isolated, enlarged, rounded cells with intranuclear inclusions; these cells appeared to be of renal epithelial origin. Yet, on using 13th passage material (titer $10^{-4.6}$ in foreskin) as the inoculum in a subsequent experiment, no evidence of infection could be found in several preparations fixed and stained after 16 days. In a single experiment employing HeLa cells, again no lesions were observed in the cultures, although rare cells with suggestive nuclear changes were found on examination of stained slides prepared on the 27th day of incubation.

TABLE I. Infectivity Titers for Different Human Tissues of Pooled, Centrifuged Fluids from Davis Cultures.

Fluid material	Days stored in CO_2 box	Tissue utilized in titration	Titer
13th pass., 14 day	0	Foreskin	$10^{-4.6}$
	11	"	$10^{-3.7}$
	11	Emb. S-M*	$10^{-4.6}$
16th pass., 17th day	4	"	$10^{-3.3}$
	42	Foreskin	$10^{-2.7}$
	42	Testis	$10^{-1.5}$

* Embryonic skin-muscle tissue.

Non-human tissues: Cultures of mouse and of chick embryonic tissues, of monkey kidney and of rabbit testis were inoculated with Davis material. No evidence of growth of the agent in these tissues was noted.

(3) *Other properties*: No evidence of ill-

ness followed intravenous inoculation of rabbits with the Davis agent and intraperitoneal and intracerebral inoculation of newborn mice with the Davis, the Kerr and the Esp. strains. Heating of Davis material in the form of cell-free fluid of known titer ($10^{-2.0}$) for 30 minutes at 56°C resulted in complete loss of infectivity. In 2 experiments, the Davis agent from the higher passages showed a 10-fold decrease in titer on freezing and thawing when tested before and after storage in a dry ice chest. Once frozen at –50°C, infectivity appeared to be well maintained. Filtration of high passage Davis virus through a sintered glass filter that retained *Serratia marcescens* was accomplished in the cold room employing a pressure of 27 mm of Hg for 15 minutes; characteristic cytopathic changes developed in cultures inoculated with the filtrate.

d. *Serologic tests*: With the availability of pools of Davis virus of known titer, a reproducible neutralization procedure became feasible. The amount of virus employed per culture in different experiments was varied as indicated and was calculated to take into account the slightly different sensitivities of the two tissues, human embryonic skin-muscle and foreskin, used in the experiments. Sera were inactivated (56°C/30 min.), and diluted with culture medium. Aliquots were then mixed with dilutions of virus, and the serum-virus mixtures kept for one hour at 5°C. Three cultures were inoculated with each serum-virus dilution using 0.2 ml amounts, 2 with each serum dilution alone, and in each experiment 3 cultures received virus alone. In screening tests, a final serum dilution of 1:10 and approximately 100 ID_{50} of virus were used; the actual amounts of virus employed in the screening studies ranged from 25 to 273 ID_{50}. All cultures were observed for a 28-30 day period. A serum was recorded as "positive" only if all 3 cultures in the group failed to develop cytopathic changes during the 28-day observation period; conversely, the appearance of specific viral activity at any time during the period in all three tubes was arbitrarily recorded as "negative." Cytopathic change in a single culture of a group was termed "? positive" and the converse "? negative." Employing this technic, serum specimens from the Davis and Kerr babies and from their mothers were examined for neutralizing antibodies to the Davis virus (Table II). Utilizing 50 ID_{50} of Davis virus, serum from the Esp. baby was positive at a 1:100 dilution.

TABLE II. Neutralization of Davis Virus by Sera from Davis and Kerr Cases.

Patient	Serum collected at age:	Virus employed in test	Results at serum dilutions
Baby D	6 mo	1250 ID_{50}	Pos. 1:100
		125 "	Pos. 1:500; Neg. 1:1000
	11 "	125 "	Pos. 1:100; Neg. 1:500
Mrs. D	19 yr	1250 "	Neg. 1:10
(Mother, 11 mo post-partum)		125 "	Pos. 1:10; Neg. 1:50
Baby K	9 days	231 "	Pos. 1:10
	26 "	25 "	Pos. 1:10; ? Pos. 1:100
Mrs. K	18 yr	25 "	Pos. 1:10
(Mother, 9 days post-partum)			

Screening neutralization tests were performed on sera from 7 additional clinical cases of cytomegalic disease. Specimens from 4 patients (ages 23 days to 3 10/12 yrs.) were positive, and three were negative or questionably so. Sera were tested from mothers of 2 of the infants in the negative group; both contained neutralizing antibodies. The case well documented by Margileth(4) gave results of this type; sera from his patient, collected at 6 weeks and at 23 months of age, were negative; those collected 4 weeks and 23 months post-partum from the mother were positive.

Sera were supplied by Dr. Rowe from 2 children whose adenoid tissues had yielded an inclusion-producing virus(7); both were negative in the screening test. Sera from 4 children, with toxoplasma-like syndromes but with negative dye test titers on examination by Dr. Feldman, were also negative for Davis neutralizing antibodies. Materials from 19 children at the Wrentham State School (ages 1 to 12), selected on the basis of the presence of "idiopathic" brain damage, were similarly

TABLE III. Results of Neutralization Tests on Sera from Unselected Individuals.

Age group (yr)	Total in group	Results: Pos.	? Pos.	? Neg.	Neg.
0- 5	28	2	1	3	22
6-18	12	1	1	0	10
19-36	25	5	1	3	16
37+	23	6	1	5	11

studied; 17 were negative, 1 ?-negative, and 1 was positive.

Additional sera were examined from healthy individuals, and from patients with a variety of illnesses. The findings are summarized in Table III. Three separate lots of human gamma globulin prepared from blood collected in Massachusetts by the Massachusetts Division of Biologic Laboratories were investigated; all were positive or "? positive" when tested in a dilution of 1:100 with Davis virus.

e. *Relationship of the Davis, Kerr, and Esp. agents to other viruses*: The Davis, Kerr, and Esp. agents did not induce the rapidly spreading cytopathic changes observed with strains of herpes simplex virus *in vitro* and also failed to produce illness in newborn mice. Furthermore, six convalescent sera from patients with herpes simplex infections failed to neutralize the Davis agent.

While the focal cytopathic changes produced by the Davis, Kerr, and Esp. viruses superficially resembled those associated with varicella-zoster viruses, consistent differences were apparent. The foci produced by the former agents were more prominent than those seen with varicella virus. Pigment deposition of the type here described has not been observed in varicella cultures. In the course of the cultivation of 21 strains of varicella virus and of 4 from patients with herpes zoster, at no time has generalized spread of the cytopathic process been observed, and virus has not been demonstrable in the cell-free fluids from infected cultures. In addition, no neutralizing antibodies to the Davis agent were found in six sera known to contain varicella antibodies.

Discussion. The morphologic changes induced *in vitro* by the Davis, Kerr, and Esp. agents suggest the relationship of these viruses to those isolated by Smith(6) and by Rowe and his coworkers(7), a concept further supported by the serologic studies of the latter investigators. It would appear that a new group of human viruses, perhaps ubiquitous in distribution, is being defined. If these agents are common associates of man, their etiologic role in entities such as the cytomegalic syndrome may be difficult to define. Considerations of this nature, together with knowledge of the occasional variable behavior of the agent *in vitro,* led us to the neutralization procedure employing a known viral inoculum and a prolonged observation period. It is possible that this method failed to detect low levels of antibody. For example, if the criterion of delay in appearance of cytopathogenicity had been employed as the index of neutralization, 6 of the 7 sera from cytomegalic cases would have been positive. However, the results of our serologic studies in general parallel those obtained by Rowe, and the finding of neutralizing antibody in pooled human gamma globulin further suggests that specific antibody is commonplace in the adult population.

The etiological import of the isolation of these agents during life can not be defined at present. The recovery of the two viruses from liver biopsy material perhaps is significant when considered in the light of recent experience. We have unsuccessfully attempted isolation of virus *in vitro* from 8 liver biopsy specimens and 6 specimens of liver obtained post-mortem; these specimens derived from a variety of pathological entities, and included one fatal case of "cytomegalic disease." The continued excretion of virus in the urine for 91 days by the Kerr infant is of considerable interest. Also, the diffuse process induced *in vitro* at isolation of the Esp. agent suggests that the urine of this patient contained relatively large amounts of virus. The demonstration of viruria, therefore, may acquire value as a diagnostic procedure. Yet it is to be noted that we have failed to isolate virus from the urine of one patient who was excreting apparently typical "cytomegalic" cells.

Summary. Viruses that produce similar

cytopathic changes *in vitro* characterized by presence of intranuclear inclusions have been isolated from 3 infants during life. The first derived from a liver biopsy from a 3-months-old child (Davis) with microcephaly, persistent jaundice, and hepatosplenomegaly; cytomegalic cells were demonstrated in the liver specimen. From a second child, virus was isolated from a liver biopsy, and on three occasions between the 14th and 91st day of life from the urine. This infant had jaundice and hepatosplenomegaly. More recently, an agent was recovered from the urine of a third infant, who evidenced hepatosplenomegaly, cerebral calcification and chorioretinitis. The Davis strain has been propagated in human fibroblasts for 20 passages during an elapsed period of 494 days. Neutralization tests with the Davis agent have indicated that neutralizing antibodies occur frequently in children with cytomegalic disease but also are not uncommon in normal adults. The agents are apparently related to those recovered in other laboratories from a human salivary gland, from a fatal case of cytomegalic disease, and from spontaneously degenerating tissue cultures of human pharyngeal tonsillar tissue.

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Anticonvulsant Properties of *l*-Glutamine and *l*-Asparagine in Mice and Rats.* (22842)

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Because of the considerable experimental and clinical interest in *l*-glutamine and *l*-asparagine as potential therapeutic agents in epilepsy, based largely on the laboratory and clinical studies of Tower(1), it was thought important to screen these agents for anticonvulsant activity in mice and rats by a battery of laboratory tests. The results obtained provide the basis for this report.

Methods. Adult male mice (CF #1 strain) from the Carworth farm and adult male albino rats from the Sprague-Dawley farm were used as experimental animals. They were maintained on Purina Laboratory Chow and, except for the experiments in rats, were allowed free access to food and water until removed from their cages for testing. The *l*-glutamine and *l*-asparagine were administered either orally or intraperitoneally in aqueous solution or suspended in 10% acacia mucilage. Both compounds were tested *in mice* after *acute* administration by a battery of six anticonvulsant assay procedures: maximal electroshock seizure pattern (MES); maximal Metrazol seizure pattern (MMS); minimal electroshock seizure threshold (MET);

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hyponatremic electroshock seizure threshold (HET); "psychomotor" seizure threshold (PsM); and Metrazol seizure threshold (Met.). Except for the fact that a Grass Stimulator (Model S4B) is now used for the PsM test, the details of these tests and the special features of the electroshock apparatus have been described in detail elsewhere (2,3, 4,5,6). Both agents were tested *in mice* after *chronic* administration by the MET and HET tests and for ability to elevate the MET lowered by cortisone. In addition, they were tested *in rats* for ability to elevate the MET lowered by food restriction. The details of these procedures have been described elsewhere(3,6,7,8). In threshold studies, the minimum current necessary to elicit facial, lower jaw and/or forelimb clonus without loss of upright posture in 50% of mice (CC_{50}) was calculated for each group by the method of Litchfield and Wilcoxon(9). Finally, *l*-glutamine and *l*-asparagine were tested for ability to prevent methionine sulfoximine-induced seizures by a method similar to that described by Tower(1).

Results. In *acute experiments,* single oral doses up to 3000 mg/kg of either *l*-glutamine or *l*-asparagine were non-toxic and were ineffective by the four electroshock assay procedures: MES, MET, HET, and PsM tests. Although 3000 mg/kg of *l*-glutamine induced 10% and 20% protection by the Met. and MMS tests, respectively, a dose of 6000 mg/kg of either *l*-glutamine or *l*-asparagine was virtually devoid of anti-Metrazol activity.

To reveal more subtle anticonvulsant effects, *l*-glutamine and *l*-asparagine were tested for *any* ability to elevate the MET, HET, and the PsM threshold. The data shown in Table I indicate that, in the dose employed, these agents are incapable of elevating electroshock seizure threshold.

Sixty rats with a mean weight of 100 g were divided randomly into 3 groups, one control group (A) and 2 experimental groups (B and C). Group C was used as the experimental control. The initial minimal electroshock seizure threshold (CC_{50}) for groups A, B, and C were 15.2 (14.4-16.5), 16.2 (15.0-17.4), and 15.5 (14.0-17.1) mA, respectively.

TABLE I. Effect of *l*-Glutamine and *l*-Asparagine on Electroshock Seizure Threshold.

Threshold test	CC_{50}*		
	Control	*l*-glutamine†	*l*-asparagine†
MET	4.5 (4.3– 4.8)	4.4 (4.0– 4.8)	4.6 (4.2– 5.0)
HET	2.4 (2.2– 2.7)	2.5 (2.2– 2.9)	2.7 (2.4– 3.1)
PsM	14.4 (13.5–15.4)	16.3 (15.1–17.6)	15.4 (14.4–16.5)

* Expressed in mA for MET and HET; in volts for PsM.
† 3000 mg/kg orally.

The control group was allowed food *ad lib.*, whereas the food intake of the 2 experimental groups was limited to 6 g/animal/day for the first 27 days and then 8 g/animal/day for the remainder of the experiment; all groups were allowed water *ad lib.* After 63 days, the mean weights were 314, 130, and 143 g for groups A, B, and C, respectively. The mean CC_{50}s were 22.8 (21.8-23.9), 15.1 (14.0-16.6), and 13.8 (12.6-15.1) mA for groups A, B, and C, respectively. Neither acute nor chronic administration of 1000 mg/kg of *l*-glutamine or *l*-asparagine had any significant effect on the CC_{50} for group A or group B. In contrast, phenobarbital (20 mg/kg, i.p.) significantly increased the CC_{50} of both groups.

The LD_{50} and 95% confidence limits of methionine sulfoximine (i.p. in aqueous solution) were determined to be 218 (194-244) mg/kg. In order to produce convulsions in nearly all animals, 30 mice were injected intraperitoneally with the LD_{97} (350 mg/kg) and at the previously determined time of seizure onset (4 hours after methionine sulfoximine) 10 mice were injected i.p. with 10 mM/kg of *l*-glutamine and 10 with a similar dose of *l*-asparagine; the remaining 10 mice were injected with the requisite volume of saline and served as controls. All animals (test and control) exhibited seizures and, except for the survival of one *l*-asparagine-treated animal, all died within 48 hours. In another experiment, pretreatment with 10 mM/kg of *l*-glutamine or *l*-asparagine 30 minutes *prior* to the methionine sulfoximine injection and again at the anticipated time of seizure onset failed to alter seizure incidence

or to increase the survival rate. Finally, neither *l*-glutamine nor *l*-asparagine had any significant effect on the seizure incidence or the LD_{50} for methionine sulfoximine.

In *chronic experiments,* the 2 agents were given orally in a dose of 500 mg/kg twice daily and tested for ability to antagonize the electro-shock-threshold-lowering effects of cortisone and hyponatremia. In the *cortisone* experiments, the minimal electroshock seizure threshold (CC_{50}) of 120 mice was determined and the animals were randomly divided into 4 groups. Two groups served as controls, one to show the response of normal animals and the other the response to cortisone (0.5 mg/animal/day s.c.). The remaining 2 groups were also given cortisone, but one group was given intraperitoneally 250 mg/kg of *l*-glutamine twice daily and the other the same dose of *l*-asparagine. The CC_{50} was determined for each group after 2, 5, and 8 days of drug treatment. Cortisone significantly reduced the CC_{50} of both the treated and the control groups. Thus, at the end of the experiment, the CC_{50} in normal, cortisone-treated, *l*-glutamine-cortisone-treated, and *l*-asparagine-cortisone-treated mice were 7.7 (7.1-8.4), 5.6 (4.8-6.5), 4.8 (3.6-6.3), and 3.8 (2.7-5.3) mA, respectively. The chronic administration of *l*-glutamine and *l*-asparagine, therefore, does not antagonize the threshold-lowering effects of cortisone. In the *hyponatremia* experiments, the experimental design was identical to that employed for cortisone except that the amides were given for 12 days. The CC_{50}s in normal, hyponatremic, *l*-glutamine-hyponatremic, and *l*-asparagine-hyponatremic were 7.0 (5.6-8.7), 5.0 (3.7-6.7), 4.6 (3.8-5.5), and 4.9 (4.2-5.7) mA, respectively. Thus, *l*-glutamine and *l*-asparagine had no significant effect on minimal electroshock seizure threshold lowered by hyponatremia.

It is concluded that *l*-glutamine and *l*-asparagine, administered acutely or chronically in the doses indicated, are devoid of significant anticonvulsant activity as measured by a variety of procedures in mice and rats.

Discussion. Tabachnick and coworkers[‡] reported that *l*-glutamine and *l*-asparagine exhibited "little or no anticonvulsant effect" as measured by the MES and Met. tests; Ginsberg(10) observed an increase in audiogenic seizure incidence in mice; and Woodbury[†] found that *l*-glutamine failed to prevent carbon dioxide-induced withdrawal seizures, although the dose of *l*-glutamine employed restored the brain glutamic acid level, previously lowered by carbon dioxide, to normal. Such observations and the data presented herein contrast sharply with the report by Tower(1) that these agents abolish methionine sulfoximine-induced seizures in an "unknown" strain of mice.

All clinically effective antiepileptic drugs can be shown to have anti-convulsant activity by one or more of the tests employed in this study. In addition, many metabolic, endocrine, and physiological factors known clinically to exert a restraining or precipitating effect on seizure susceptibility have been shown in our laboratory to influence brain excitability as measured by these same tests. For example, such clinically important factors as pH changes, ketosis, plasma pCO_2, starvation, pyridoxine, adrenocorticosteroids, water, and electrolytes—known to influence the occurrence of various types of seizures—have been shown by us to exert predictable changes in seizure susceptibility in laboratory animals by procedures similar to those used to study *l*-glutamine and *l*-asparagine. Hence, if controlled clinical studies eventually indicate any efficacy of these amides in the treatment of epilepsy, the mechanism of their action must be considerably different from that of known anticonvulsant drugs, metabolic factors, hormones, or nutrilites.

Summary. Two amides, *l*-glutamine and *l*-asparagine, were tested for anti-convulsant activity in mice after *acute* administration by a battery of 6 anticonvulsant assay procedures and after *chronic* administration by 3 tests. In addition, both agents were tested in mice for ability to prevent methionine sulfoximine-induced seizures and in rats for ability to elevate the minimal electroshock seizure threshold lowered by food restriction. The results indicate that both these agents are vir-

‡ Reported in *Fed. Proc.,* 1956, 490.

† Unpublished observations.

tually devoid of anticonvulsant activity as measured by a number of diverse laboratory screening procedures.

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D_2O Equilibration Rates in Thermally Injured Animals.* (22843)

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There have been numerous attempts to make *in vivo* dissections of the human body to understand better the phenomena which occur in both normal and pathologic states. Isotopes have facilitated this work; but no isotope is ideal since each has its own peculiar advantages and disadvantages. Most investigators consider deuterium oxide the isotope of choice in measuring total body water. The principle of isotope dilution is well known and accepted; and it is dependent upon knowing the amount of substance injected, amount of substance excreted prior to equilibrium, and concentration of this substance in the body at equilibrium. In the majority of cases, equilibrium of D_2O in the normal state will be attained in 1 to 2 hours. Some investigators have allowed 3 hours for D_2O to equilibrate in certain pathologic states; thus they imply some delay in equilibration(6). Others insist that time to equilibrium is even longer(5). This is particularly true with ascites.

It is most important to determine total body water in any study of profound alterations in fluids and electrolytes following thermal injury. Determinations of total body water are also used with determinations of extracellular fluid space, to measure intracellular water compartment. It has been questioned as to whether or not D_2O would equilibrate within a 2- or 3-hour period in subjects when there were large edematous areas as a result of thermal injury. For this reason, equilibration studies were made on thermally-traumatized animals to determine whether or not the time to equilibrium was prolonged. Each animal acted as its own control.

Materials and methods. Sixteen adult mongrel dogs were used, average weight 16.8 kg, ranging from 12.7 to 23.0 kg. Each animal was weighed 3 times a week until its weight had stabilized, usually in 4 to 6 weeks. Food was withheld from animals for 12 hours prior to experiment; however, all animals had free access to water until 4 hours prior to experiment. Dogs were anesthetized with intravenous phenobarbital anesthesia (30 mg/kg). Femoral vessels were exposed through small incision in the groin and both femoral artery and femoral vein were cannulated with polyethylene tubing. Sterile D_2O (purity greater than 99.5%) as isotonic saline was obtained in 50 g ampules (Abbott). Twenty cc of D_2O-saline solution were injected into femoral vein through a calibrated syringe. The polyethylene tubing was rinsed repeatedly with

* The author appreciates assistance of Hubert Lindsay.

TABLE I. Comparison of D_2O Equilibration Rates in 16 Thermally Injured Animals.

Pre-injury				Post-injury				Diff. in time to equilibrium
Wt (kg)	Vol of distribution	% body wt	Min. to equilibrium	Wt (kg)	Vol of distribution	% body wt	Min. to equilibrium	
15.0	8.92	59.47	240	15.6	10.60	67.95	150	− 90
14.3	8.20	57.34	90	16.3	7.53	46.20	90	0
16.7	10.85	64.97	180	16.7	11.03	66.25	180	0
14.8	9.17	61.96	150	13.3	9.81	73.76	150	0
14.1	8.77	62.20	90	14.7	9.36	63.67	240	+150
18.2	9.71	53.35	90	18.7	10.91	58.34	90	0
16.9	10.94	64.73	150	17.5	10.64	60.80	90	− 60
12.7	6.94	54.65	150	13.9	6.85	49.28	150	0
17.2	11.51	66.92	150	17.8	12.03	67.58	150	0
19.7	9.62	48.83	90	19.1	10.38	54.35	150	+ 60
16.0	9.60	60.00	90	19.5	9.66	49.54	180	+ 90
22.5	11.83	52.58	150	22.1	13.41	60.68	150	0
23.0	15.77	68.57	90	24.1	17.39	72.16	180	+ 90
19.9	13.87	69.70	90	21.1	14.00	66.35	90	0
14.8	9.87	66.69	90	14.5	10.74	74.07	150	+ 60
13.3	7.88	59.25	180	13.1	7.91	60.38	180	0
Avg 16.8	10.22	60.70	129.4	17.37	10.77	61.96	148.1	18.75
		S.D. ± 46.11				S.D. ± 41.67		

small amounts of normal saline. Blood samples were taken from femoral artery at 90, 150, 180, 240, 270, and 300 minutes following injection. Blood samples were collected in small glass ampules, flame-sealed immediately after collection. At least 3 weeks elapsed between studies. In some cases the interval was as long as 8 weeks. The animals were anesthetized again in identical manner as previously. A standard, body-surface injury was produced by immersing the lower half of the animal to a point midway between groin and umbilicus in water at 90°C for 20 seconds. Following thermal injury, animals were observed for 8 hours during which time no fluid therapy was administered. Minimal amounts of pentobarbital were given to keep animals comfortable. *Femoral vessels* in unoperated leg were exposed and cannulated at the end of 8-hour waiting period, the cannulae being threaded well up into the common iliac vessels. Twenty cc of D_2O-saline solution were injected into the femoral vein from a calibrated syringe. Blood samples were collected from femoral artery catheter by the same method and time intervals as those in the initial study. *Plasma samples* were prepared by vacuum-distillation technic and the D_2O levels of these samples were determined by the falling drop method(2).

Results of the experiment are summarized in Table I. The average weight of animals in the preburn series was 16.8 kg; in postburn series 17.4 kg. The smallest animal weighed 12.7 kg. Smaller animals were not used because the mortality rate during postthermal injury 8-hour waiting period was much greater than in larger animals. Weight variation of each animal was directly related to length of time between the two procedures, *i.e.*, dog No. 11 gained 3.5 kg in 2-month interval.

The average D_2O volume of distribution was 10.32 l in the preburn series and 10.77 in the postburn series. Using the paired "t" test, a statistical analysis was made on differences of columns of distribution between preburn and postburn series using animals as their own controls. There was no statistically significant difference. The standard deviation of differences of volumes of distribution was 0.707 liter. The standard deviation of the mean of the differences was 0.177 liter. Replicate determinations of volumes of distribution of D_2O in normal animals have been made in this laboratory and the standard deviation was 0.510 liter. The time interval between these replicate determinations ranged from 3 to 8 weeks.

In this experiment, volume of distribution as per cent of body weight was 60.7 in the preburn series and 61.96 in the postburn se-

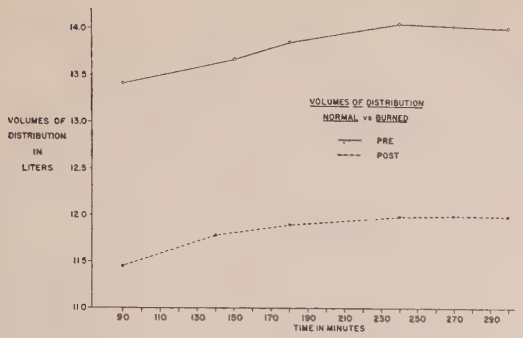

FIG. 1.

ries. Using the paired "t" test, a statistical analysis was made. There were no significant differences between volumes of distribution recorded (as per cent of body weight) between preburn and postburn series. The standard deviation was 6.67% of body weight.

D_2O concentration in the plasma specimen was determined; and volume of distribution was calculated at each time interval. The volumes of distribution were plotted on arithmetic paper. The time to equilibrium was selected as that interval when volume of distribution was within 5% of final volume of distribution at 300 minutes (Fig. 1).

The average time for D_2O to equilibrate in the preburn animal was 129.4 minutes. Standard deviation was 46.11 minutes. The time necessary for equilibration was somewhat longer in the postburn series. The average time was 148.1 minutes, with standard deviation of 41.67 minutes. Using the paired "t" test, a statistical analysis disclosed no statistically significant differences between the time necessary for equilibration of D_2O in preburn *vs.* the postburn series. The standard deviation of differences of time to equilibrium was 58.9 minutes.

Discussion. Schloerb *et al.*(4) found that equilibration was complete in 2 hours in the normal human. Edelman *et al.*(8) studied the time necessary for D_2O to penetrate a human bone and to come into equilibrium with the serum and found that it took approximately 4 hours. Thus, there is apparently no difficulty for D_2O to penetrate remote and inaccessible areas of the normal body and to equilibrate with these areas with considerable rapidity.

Since 1950, most investigators have assumed that equilibration was complete in 2 or 3 hours regardless of the pathologic state (6). The possibility that sampling at 3 hours did not allow adequate time for equilibration was suggested by Last *et al.*(9) who found greatly increased time to equilibration using inulin and mannitol in edematous states. Faller *et al.*(5) showed that the time to equilibrium for D_2O was prolonged in several pathologic states particularly in ascites. Edema fluid following a burn is entirely different insofar as the D_2O has a greater opportunity to reach equilibrium with the edema fluid because the area for diffusion in comparison to the pericapillary area is much greater than in the case of ascites with peritoneal surface. In this experiment the time required for equilibration in both unburned and burned animals showed considerable variation, range being 90-240 minutes. The average time for equilibration in the unburned animal was 129.4 minutes while in the postburned animal it was 148.1 minutes. There was no statistical significance between the time required for equilibration between animals in preburn and postburn series. The variation in time for equilibration should be stressed in order to show that more than 2 hours is necessary for equilibration in any study involving the D_2O analysis.

The postburn animals had virtually the same volume of distribution as in the normal state. The 1.5 l variation as shown in Fig. 1 was somewhat greater than in the majority of cases since the standard deviation of the differences was 0.707 liters. Because of the severity of the burn there were marked changes in the blood picture. All blood samples showed marked hemolysis of red cells, and the hematocrit postburn varied between 60 to 75%. The average volume of distribution of D_2O as per cent of body in the normal animal was 60.7(2,5).

From the above findings it appears that 2 hours for equilibration is inadequate. While 3 hours is adequate, in most instances, blood samples should be taken for 5 hours because of considerable variation in circulatory efficiency in normal and pathologic states.

Equilibrium cannot be assumed to be attained in either normal or pathologic states unless consecutive blood samples show little or no variation either in volumes of distribution or concentration of D_2O. It is the normal variation, rather than edema formation following burns, which necessitates a longer time interval for equilibration.

Summary. 1. The average time to equilibrium following intravenous injection of D_2O in 16 normal adult mongrel dogs was 129.4 minutes. Equilibrium was established in thermally injured dog in 148.1 minutes. There were no statistically significant differences between animals before and after thermal injury. 2. Equilibrium should not be assumed to be complete at 2 hours in either normal or pathologic states. Consecutive blood samples should be taken over a longer period of time, preferably up to 5 hours. A point of equilibrium should be selected when variation is within 5% of final volume of distribution at 5 hours. 3. Because of wide variation in time to equilibrium in both normal and pathologic states, any conclusions regarding changes in "efficiency of total circulation" should be studied carefully and interpreted cautiously.

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Effects of Rhythm and Rate Changes on Inotropic Action of Ouabain.* (22844)

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These studies were performed to determine the relationship of changes in rhythm and rate to the inotropic action of digitalis glycosides. In the experiments on rhythm, the relationship of the inotropic action of ouabain to postextrasystolic potentiation was studied. This latter phenomenon was first described by Woodworth(1) in the dog heart. He found that when an extra contraction was interpolated in a series of regular contractions, the regular contraction immediately following the extra contraction was augmented. Cattell and Gold(2,3) subsequently redirected attention to this phenomenon and clarified many of its aspects. Recently several other studies of this response have been reported(4-8) in both atrial and ventricular musculature of different species. The term postextrasystolic potentiation was first used by Hoffman *et al.*(8). The occurrence of postextrasystolic potentiation indicates that there are sources of muscular power which can be demonstrated by alterations in the pattern of stimulation, and it was of interest to determine whether the inotropic action of a glycoside involved these sources of energy. In the second part of the experiment, the re-

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lationship of the glycoside action to the rate of stimulation at regular frequencies was investigated. There is a significant latent period after administration of a glycoside before an increase in force is seen, and a longer period before maximum effect is produced, although there are differences between individual glycosides. The duration of the latent period may be related, either to the number of contractions which take place before the glycoside effect is seen, or to the amount of time which elapses.

Method. The cat papillary muscle technic of Cattell and Gold(9) was used, with a phosphate-buffered Ringer's solution containing glucose, 1.8 g/L and, bubbled continuously with oxygen. In the first part of the study, the 14 preparations were stimulated at a base rate of 30 per minute, and an extra beat interpolated at intervals of 250, 300, 350, 400, 450 and 500 milliseconds, using the technics described earlier(5,7). The effects of rest periods of 10 seconds, with and without the interpolated beat were recorded and measured. These measurements were repeated after failure of the contractile force, and upon recovery after ouabain, 1 to 10 million. In analysing the data, all changes were compared to the initial control values to make possible a ready differentiation between changes due to a modification of the postextrasystolic potentiation and changes produced by the failure or recovery alone. In the second part of the experiment, 2 muscles were removed from each heart, and set up in separate chambers. One muscle of a pair was stimulated at a rate of 12 per minute and the other at a rate of 60 per minute. After contractile force had diminished to about half of the initial value, ouabain was added to each preparation to produce a concentration of 1:5 million. Measurements of contractile force were made at 5 minute intervals thereafter. In these experiments, 28 muscles were used.

Results. In the studies on postextrasystolic potentiation, the degree of potentiation fell gradually during the period of failure, and continued to fall despite the recovery of the muscle under the influence of ouabain

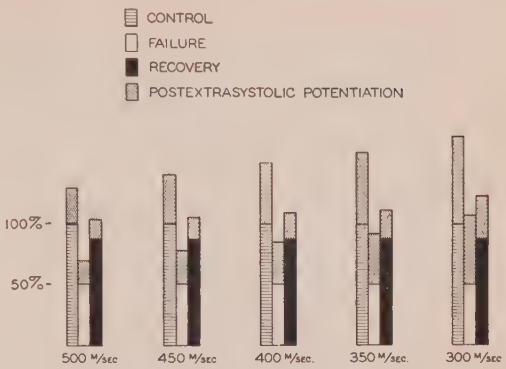

FIG. 1. Relationship of postextrasystolic potentiation to failure, and ouabain-induced recovery.

(Fig. 1). Similar results were noted at all the interpolation intervals, although, as reported earlier(5,7) the shorter the interval, the greater the potentiation. As an example, the results obtained at an interpolation interval of 350 m/sec may be considered. During the control period, postextrasystolic potentiation was 58% (S.E. 10.3, $N = 8$) of control force. After the failure of the regular contractions to 50% of the control (S.E. 3.7, $N = 8$) the postextrasystolic potentiation was 42% (S.E. 7.7, $N = 8$). After ouabain, there was an average recovery of regular contractile force to 87% of the control (S.E. 8.8, $N = 8$). At the peak of this recovery, however, the postextrasystolic potentiation had fallen to 24% (S.E. 6.4, $N = 8$). At any individual interpolation interval, the difference between postextrasystolic potentiation during the control interval and at the time of failure, and the difference between the potentiation at failure and after recovery are not statistically significant. However, since similar results were seen at all 5 interpolation intervals (Fig. 1), it is likely that these changes are meaningful. The major findings, which are apparent from Fig. 1 are that the rate of failure of the regular contractions differs from the rate of failure of the postextrasystolic potentiation, and that ouabain does not improve postextrasystolic potentiation.

In the experiments on differing rates, the speed of failure at a stimulation rate of 60 per minute was found to be only slightly greater than at 12 per minute. At the slower

stimulation rate, it took an average of 215 minutes for the muscles to fail to 39.5% of maximum force (S.E. 4.0, N = 14). At the more rapid stimulation rate, it took an average of 172 minutes to fail to 39.5% of maximum force (S.E. 2.5, N = 14). The muscles were observed continually, and it was clear that the time course of failure was similar in the two series. No significant differences were seen. After the addition of ouabain, the contractile force improved at a slightly faster rate in the muscles stimulated at 12 times per minute. At 60 minutes after ouabain they had improved 23.1% (S.E. 4.4, N = 13) while the more rapidly stimulated muscles improved 20% (S.E. 3.5, N = 14). At 90 minutes, the improvement was 33.7% (S.E. 6.2, N = 12) for the slowly stimulated preparations and 27.0% (S.E. 6.0, N = 13) for the rapidly stimulated preparations. At 120 minutes, the improvement was 44.5% (S.E. 7.7, N = 13) for the slowly stimulated preparations, and 32.2% (S.E. 7.0, N = 13) for the rapidly stimulated preparations. The average maximum improvement attained at the stimulation rate of 12 per minute was 56.5% (S.E. 5.7, N = 14) in an average of 141 minutes. At the faster rate, the maximum improvement was 42.9% (S.E. 6.1, N = 14), reached in an average of 138 minutes.

Discussion. Since ouabain produced significant improvement in the force of the regular contractions after failure, but did not produce any improvement in postextrasystolic potentiation, it may be that in the latter phenomenon, additional contractile mechanisms are involved.

Rosin and Farah(4) and Katzung and Farah(6) have postulated the existence of a "potentiating substance" to account for postextrasystolic potentiation. Our experiments neither confirm nor contradict their hypothesis, but do suggest that if such a substance is present, its production gradually falls off in the isolated tissue, and is unaffected by ouabain.

The rate experiments suggest that the latent period in glycoside action on mammalian heart muscle is related to elapsed time, rather than to the number of contractions. The muscles stimulated at a rate of 12 per min. improved 23.1% in 60 minutes, or 720 beats, while the muscles stimulated at a rate of 60 per min. improved 20.0% in 60 minutes or 3,600 beats. If the latent period were related to number of contractions, the improvement after 3,600 would be significantly greater than after 720 beats. Since this is not the case, it is probable that the elapsed time is the important factor in the latency of the glycosides. Although there are differences between the two series they are slight, and not at all in the same proportion as the rate difference, which was 5 to 1. The small differences observed in these experiments probably result from slightly more rapid failure at rapid than at slow stimulation rates. Wilbrandt *et al.*(10) studying the frog heart arrived at different conclusions. They found that under the conditions of their experiments, the time required for glycoside effect depended on the number of contractions. In their studies, they used a calcium-poor solution. The difference between their findings and those reported here may result from the different solutions used, or from a difference in species. It has been pointed out several times(7,11,12) that there are marked differences between the responses to drugs of frog and mammalian hearts.

Summary. In failing cardiac muscle, postextrasystolic potentiation decreases at a slower rate than response in a series at a regular frequency. Postextrasystolic contraction is not improved by ouabain. The latent period of ouabain action is related to elapsed time, not the total number of contractions.

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Agglutination of Blood Cells in *Limulus polyphemus.** (22845)

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The horseshoe or king crab, *Limulus*, is as distinctive among the arthropods in its pattern of hemostasis as it is in other aspects(1). It appears to have neither fibrinogen nor the power of autotomy, relying on the toughness of its integument and a very large number of agglutinating cells to prevent blood loss. Not only does it possess many more of these cells than other arthropods(2), but the formation of the solid mass seems to differ qualitatively. Two cellular phases, agglutination and "gelation," have been distinguished(3), the latter of which is distinct from the process of gelation by polymerization of plasma fibrinogen which occurs in crustacea.

It has been difficult to study this phenomenon since procedures which stabilize vertebrate or even crustacean bloods—cooling, removal of calcium ions, addition of heparin, provision of hydrophobic surfaces, etc.—are largely ineffective in *Limulus*(4). By working quickly in the cold, both *Limulus* and crustacean cells can be separated before agglutination occurs but under these circumstances it is not possible to resuspend the centrifuged mass of cells. Agglutination is effectively prevented by formaldehyde fixation, a procedure which is used in counting blood cells or observing their structure(2). This drastic reagent would ordinarily be considered incompatible with any functional aspects but it was found to be effective at relatively low concentrations (0.05-0.10%). Higher concentrations (0.25-0.50%) resulted in some clumping and also precipitated some plasma protein. The cells separated from formaldehyde treated plasma could be readily resuspended in formaldehyde-free sea water or other salt solutions and maintained indefinitely without clumping.

However, these stable suspensions quickly agglutinated upon the addition of small quantities of *Limulus* plasma (0.01-0.05 volumes). In suspensions of 10-20 x 10^6 cells/cc this clumping began within 2 minutes and was complete in 10-15 minutes with large clumps of more than a hundred cells usually

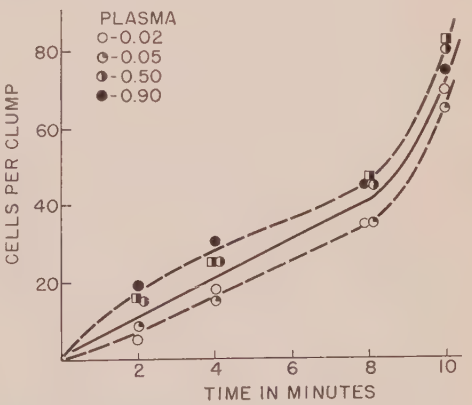

FIG. 1. The course of clumping of stabilized *Limulus* cells suspended in artificial sea water with various concentrations of added plasma; cell concentration, 14.8 × 10^6/ml; plasma concentrations as indicated (referred to whole plasma); temperature 22°; circles, fresh cells; squares, cells dried from the frozen state and resuspended. Ordinate represents avg No. of cells per clump.

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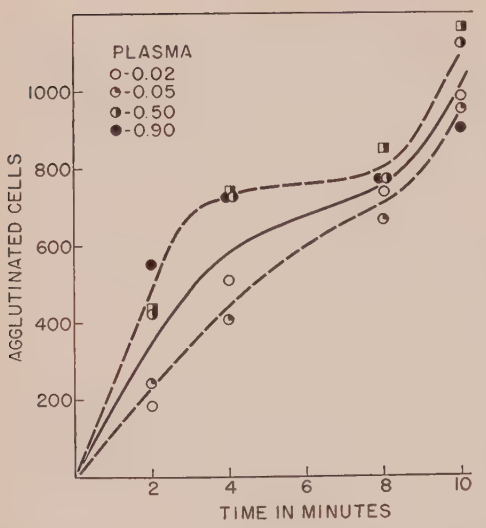

FIG. 2. The course of agglutination of *Limulus* cells at various concentrations of added plasma. Ordinate represents total No. of clumped cells in counting area. Conditions and symbols as in Fig. 1.

settling out. The time course of this reaction is shown in Fig. 1. After an initial period, the increase in clump size is largely independent of the plasma concentration over the observed range of 0.02-0.90. A similar relation in terms of the total number of cells agglutinated is shown in Fig. 2. This suggests a 2-stage process and emphasizes the initial dependency on the plasma concentration. Heterologous plasma from the spider crab (*Libinia*) was ineffective in agglutinating *Limulus* cells.

Plasma-free cell suspensions could be frozen and dehydrated to yield a friable powder which, upon resuspension, was little different from the original material in microscopic appearance. It also appeared to have lost none of its functional attributes, insofar as these interests are concerned, since agglutination quickly occurred following the addition of plasma. Indeed, as is shown in Fig. 1 and 2, the course of the reaction was quantitatively identical to that before desiccation. Suspension of the cells in distilled water prior to drying or to concentrated salt solutions after drying produced the expected aberrations in appearance—crenation or rupture—but did not prevent the cells from clumping in the presence of plasma.

The very marked effect of temperature on

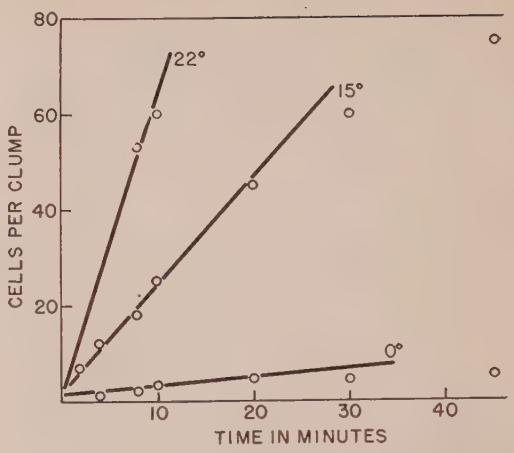

FIG. 3. The course of clumping of *Limulus* cells at 0, 15 and 22°C. Cell conc., 15.0 × 10⁶/ml; plasma conc., 0.10.

cell clumping is shown in Fig. 3. The rate of clumping was reduced by a factor of 2.7 between 22° and 15°, and by a factor of 12 between 15° and 0°. The corresponding Q_{10} values (factor for a 10° temperature increase) were 4.1 and 5.2 respectively. Like calculations referred to the number of cells agglutinated gave very similar results ($Q_{10} = 4.6$ and 5.0).

The early stages of the reaction were definitely concentration dependent as is indicated in Fig. 4 which plots the initial rate against the log plasma concentration. A linear (semilogarithmic) relation is seen which suggests

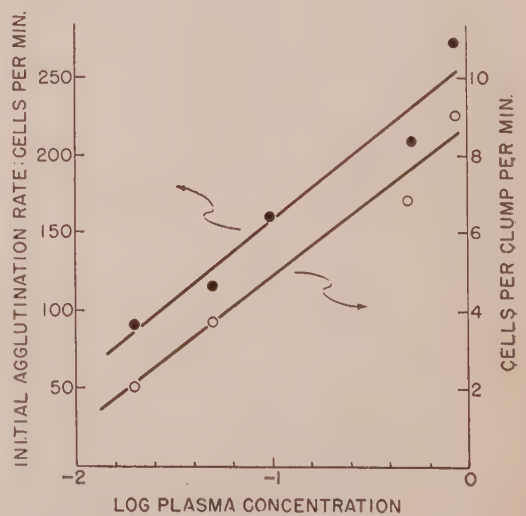

FIG. 4. Initial agglutination and clumping rates as a function of log plasma conc.

that at plasma concentrations below 0.002 no agglutination would occur.

Discussion. It is probable that formaldehyde modified the cell surfaces even though they could still be agglutinated once the free agent was removed since the second "gelation" stage was never observed in these stabilized systems. The effect on the plasma factor was more drastic since even after dialysis, the formaldehyde-treated plasma could not agglutinate the resuspended cells. Formaldehyde also very effectively prevents the interaction of fibrinogen to form fibrin (5). Inasmuch as heterologous plasma did not cause agglutination it would appear that a special "factor" was involved rather than a non-specific effect of, say, plasma protein.

The quantitative resistance of this cellular activity to drying is of interest but is in accord with the general durability of agglutinating systems. The very high temperature coefficient of the reaction is perhaps surprising. Much lower values have been observed for the polymerization of both vertebrate and invertebrate fibrinogens(6).

Summary. Stable cell suspensions were prepared from *Limulus* blood using 0.05-0.10% formaldehyde. Washed cells could be agglutinated by as little as 0.01 volume of fresh homologous plasma. The initial stages of this reaction showed moderate concentration dependence and very strong temperature dependence.

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Evidence for an Increased Intake of Sodium in Hypertension Based on Urinary Excretion of Sodium.*† (22846)

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In other papers, evidence has been presented which indicated that salt (*i.e.* sodium) intake played a primary role in the pathogenesis of human essential hypertension(1-2). Since this thesis was developed by semi-quantitative means, confirmation by quantitative technics seemed pertinent and forms the subject of the present report. Beginning in 1953, every member of the Brookhaven National Laboratory staff who reported to the staff clinic for annual physical examination by Dr. Robert A. Love was questioned as to his salt intake and classified into one of 3 groups as follows: 1) *Low intake*—this individual did not add, and had never added, salt to his food at table; 2) *Average intake*—this individual added salt to his food if *after* tasting, it was insufficiently salty; and 3) *High intake*—this subject routinely added salt to foods customarily salted, but did so *before* tasting them. The defects in this type of classification were recognized, but the author thought this was a method by which a large series could be studied readily and the implications of a low versus a high salt intake tested. The other papers may be consulted for further details but in summary the following results were obtained: Among the 1346 adults who were classified according to the categories listed above, there were 135, 630 and 581 in

* This work was carried out under auspices of Atomic Energy Commission.

† The author wishes to thank Phelps Crump, for his helpful criticisms of the statistical analyses.

TABLE I. Average Daily Urine Sodium Values in 28 Ambulatory Male Subjects.

"Low" NaCl intake				"Avg" NaCl intake				"High" NaCl intake			
Age	Days urine collected	mEq Na per day (avg)	Hyper-tension	Age	Days urine collected	mEq Na per day (avg)	Hyper-tension	Age	Days urine collected	mEq Na per day (avg)	Hyper-tension
48	9	197	+	33	7	210	0	45	7	244	+
40	6	173	0	69	7	202	+	25	7	236	0
33	7	171	0	37	8	191	0	33	7	236	+
43	7	158	0	33	28	181	0	42	7	227	+
39	7	157	0	40	38	180	0	36	7	202	+
49	7	140	0	56	7	179	+	31	7	195	0
55	13	126	0	35	8	134	0	53	7	192	+
36	7	125	0	29	7	131	0	27	7	185	0
				43	7	116	0	45	7	184	0
								45	6	160	+
								28	7	156	0

the Low, Average and High intake groups respectively. The 105 hypertensives in this series were distributed as follows: Low intake, 1; Average intake, 43; High intake, 61. Tested by the chi-square method, the probability of this distribution occurring by chance was found to be less than one in a thousand ($\chi^2 = 16.1$, $p < .001$). Individuals classified in the Low NaCl intake group had significantly less hypertension ($p < .01$) while those on a High intake had significantly more hypertension ($p < .01$) than would have been predicted by chance alone.

The current study had as its goal the quantitation of urinary sodium among individuals with and without hypertension in the several salt-intake groups described above. In prolonged metabolic studies on ambulatory individuals both normal and hypertensive, the author has found the urinary sodium to be a highly reliable measure of the sodium intake. Therefore, while urinary excretion of sodium was measured, under the conditions of the current study it seems reasonable to assume that this accurately reflected the actual intake of this ion.

Procedure. Selection of individuals was based on: 1) Sex—only males were tested because of greater ease of collecting samples, lack of need to allow for pre-menstrual Na retention, and because analysis of data had indicated that statistical significance was unaffected by elimination of females; 2) Presence or absence of hypertension; 3) Availability and willingness to participate for approximately one week. All subjects were in essentially sedentary occupations at the Laboratory. Most of these people were unaware of the nature of the study, but included in the series are 5 colleagues from Brookhaven Medical Department who knew the general problem under investigation: all 5 denied any conscious deviation from their normal use of salt at table. Two others, however, stated that they had decreased their salt intakes consciously and therefore have been omitted. Complete 24-hour urine collections were made for 6-38 days (median 7 days; average 9.1 days) and in almost all instances, the 24-hour collections were made on consecutive days. Each collection unit contained a detailed set of directions on procedure to be followed; in case a sample was lost, the subject was instructed to discard the collection and resume on a full 24-hour basis. Each individual was told not to deviate from his normal behavior pattern and only in event of illness was the test to be abandoned. Urine was voided directly into chemically clean capped bottles. After measurement of volume from each 24-hour specimen an aliquot was removed for subsequent sodium analysis. Sodium concentration was measured in duplicate on a Baird Model DB2 flame photometer using internal lithium standard. From the 24-hour urine volume and concentration of sodium, the 24-hour urine sodium output was calculated. Since stool sodium amounts to only about 3 mEq/d(3), in the absence of unusual extra-renal losses, Na retention, or advanced renal disease, the urine Na output reflects accurately the intake of this ion(4). Because

TABLE II. Statistical Summary of Data.

By Student's t test, the mean ages in the groups did not differ significantly. By the same test, the mean level of sodium in the urine differed significantly among the following groups:

Low *vs* high $p < .01$
Low *vs* high (non-hypertensives) $p < .02 > .01$
Non-hypertensives (all groups) *vs* hypertensives (all groups) $p < .01$

	"Low" NaCl group	"Avg" NaCl group	"High" NaCl group	Total
Subjects	8	9	11	28
Age—yr*	42.9 ± 7.4	41.7 ± 10.6	37.3 ± 9.2	40.3 ± 10.6
Hypertension	1	2	6	9
Urine Na—mEq/day*				
(a) All subjects	155.9 ± 25.2	169.3 ± 33.7	201.5 ± 30.5	178.1 ± 35.2
(b) Non-hypertensives	150.0 ± 19.5	163.2 ± 35.7	191.2 ± 28.9	165.7 ± 32.0
(c) Hypertensives	197	191	210	204.3 ± 27.3

* Mean and S.D.

all studies reported here were done during cool or cold weather, it is unlikely that sweat losses were significant. There were no clinical grounds for expecting abnormal Na retention. There was no evidence of renal disease in the subjects except for mild albuminuria in some members with hypertension. Blood pressure measurements were made with mercury sphygmomanometer while patient sat quietly in upright position with arm resting on examiner's desk. Systolic and diastolic levels were assumed to be represented by the first and fourth auscultatory phases, as recommended by a Committee of the American Heart Association(5). Hypertension was defined as a level of at least 140 mm of mercury systolic in combination with a pressure of at least 90 mm diastolic.

Results. The data are summarized in Tables I and II. The most significant points to be noted are:

1) Groups of individuals who had been previously classified as having "low" salt intakes showed significantly lower ($p < .01$) average levels of urinary sodium excretion than individuals classified as having "high" salt intakes.

2) Individuals previously classified as having "average" salt intakes were presently found, as expected(1), to range throughout the low-average-high spectrum of sodium excretion. Statistically this group did not differ significantly from the other 2 groups.

3) The single individual in the "low" intake group who had hypertension, excreted sodium in amounts equal to members of the "high" salt-intake group rather than to his non-hypertensive companions in the "low" intake group.

4) When the 28 subjects were reclassified into 2 groups according to presence or absence of hypertension, it was found that the group with hypertension had significantly greater ($p < .01$) Na excretion than did those without hypertension.

Discussion. In view of the sedentary occupations of these subjects differences in activity among members of 3 groups with corresponding variations in sodium loss through sweat, could hardly account for these findings. There is no evidence that hypertensives have a different metabolic rate from non-hypertensives; and a metabolic study reported by Dole *et al.*(6) as well as observations in the author's laboratory reveal no correlation between changes in sodium intake and metabolic rate. Therefore it is improbable that the output of sodium is related to metabolic rate. In rats with experimental renal hypertension, it has been found that if self selection is permitted, a *reduction* of sodium intake usually occurs in association with development of hypertension(7). Aside from obvious differences in the species of animal in that study and the present one, it seems fair to say that human essential hypertension and experimental renal hypertension cannot be regarded as identical diseases(8). Indeed, Castleman and Smithwick have reported(9,10) that among individuals who

underwent bilateral sympathectomy for hypertension, histologic changes were absent or insignificant in biopsied kidneys of about a quarter and nearly the same fraction had only minimal changes.

The correlation between high salt intake (as intake is defined by urinary sodium excretion) and hypertension does not necessarily establish the dependency of one on the other but such a cause-effect relationship must be heavily implicated. More prolonged studies of a larger series of subjects are obviously necessary. Nonetheless, since the quantitative data in this relatively small group are in agreement with those of the 1346 individuals analyzed earlier, it is reasonable to anticipate further confirmation. If an enhanced salt intake is indeed found in hypertensives an important question is whether it is a manifestation, rather than a cause, of the disease. It has been suggested that hypertensives have an increased appetite for salt(11) a possibility which the work reported here cannot exclude. However, for 8 years the author has used drastic Na restriction (40-125 mg/day) under metabolic ward conditions for study and treatment of patients in all stages of this disease: no evidence of salt craving has been noted such as might have been expected if excessive salt appetite were part of the illness. Many of these patients had consumed very large amounts of salt prior to reduction of intake so that this seems relevant to the issue.

Conclusion. Among 28 subjects with and without hypertension, the group with high blood pressure had significantly greater ($p<.01$) sodium intakes (as measured by urinary outputs) than did the group of nonhypertensives. This finding was in agreement with a hypothesis previously proposed, namely, that the level of sodium intake was of primary etiologic importance in the development of essential hypertension.

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Effect of O-Diazo Acetyl-L-Serine on Rat Litter.* (22847)

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O-Diazo acetyl-l-serine (AZS), a serine analogue first isolated from culture broth of a streptomyces and later synthesized(2) was reported to affect adversely the chick and rat embryo(4,7,8). According to the first report(7), pregnant rats treated with a single dose of 5 mg/kg or 2.5 mg/kg doses failed to produce live litters when injected on the critical 8th day of gestation. Doses on the 10th and 11th days induced fetal resorption and malformations(7,8). The main object of the present experiments was to determine not so much the effect of the drug on the single fetus but on the total litter, its effect on repeated use in the same animal and its effect on subsequent fertility and litter.

Methods. Long-Evans strain of rats was used as previously described(9,10). The single LD_{50} for Wistar rats of 200 g weight was reported to be 70 mg/kg intraperitoneally and the LD_{50} of 5 doses on 5 successive days 25 mg/kg(7). The doses of AZS administered in the present experiments to the pregnant mothers varied from 2.5 to 10 mg/kg. Experimental groups of rats, which all had only one previous litter, were injected intraperitoneally on the 4th and 5th, the 8th, or 7th and 8th, or 11th and 12th, or 15th and 16th days and sacrificed on the 21st day of gestation (the day before littering), counting the morning of massive sperm findings in the vagina as day 0. At autopsy no abnormalities were detected in the mother rats. The entire uterus with tubes and ovaries was removed, then opened longitudinally, opposite the site of the placental implantations. The placentas, fetuses and resorptions were counted, weighed, and recorded. The fetuses were inspected, measured, weighed and then fixed in 10% formalin. All fetuses were x-rayed and the cranium incised for evidence of hydrocephalus.

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Results. Weight changes. Normal, nonpregnant control groups of rats of the same age as the experimental animals gained 8%, and pregnant controls 36%, of their initial body weight during a period of 21 days. The experimental groups of rats with their litter intact (Table I, 1,2,9) gained weight like the control rats, but groups of animals with their entire litter destroyed following drug administration on the 8th day gained an average of 18% (Table I, 6) and with drug administration on the 11th and 12th days, an average of 11.5% of total initial body weight (Table I, 8). This weight gain indicated that the doses of AZS administered were not toxic to the mothers.

Effect on litter. Two doses of 5 mg/kg AZS given intraperitoneally on the 4th and 5th days of gestation failed to show an effect on the litter (Table I). The single 2 mg/kg intraperitoneal dose which is equivalent to a 2.5 mg/kg oral dose on the 8th day of gestation, led to stunting of 11% of all the living fetuses, and to resorption of 17% of all implants (Table I, 2). The 2.5 mg/kg intraperitoneal dose permitted only 13% of all fetuses to survive, with stunting and malformation of 34% of the survivors (Table I, 3). Thirteen out of 28 mothers, or 41%, were found at sacrifice to have lost their entire litter, (Table I, 3). The same dose given on the 7th and 8th days of gestation showed no significant difference in fate of the litters at sacrifice (Table I, 4). The single 5 mg/kg intraperitoneal dose resulted in the survival of only one single malformed fetus in the entire group of 26 rats treated, the others being completely resorbed (Table I, 5). The 10 mg/kg intraperitoneal dose killed all fetuses without exception (Table I, 6). Two doses of AZS, given on the 11th and 12th days of gestation, or at midterm for rats, were found to be equally fatal to the litters. The 5 mg/kg doses permitted the survival of 8 stunted

TABLE I. Effect of AZS on Rat Litter.

No. of rats		Day of dose	Dose, mg/kg	Mother's wt gain, %	Total implants	Fate of litter: Live	d	Dead	d	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
							Stunt		Malf.					
18	a	4, 5	5.0 i.p.	+36.5	166	162		1		4				
	b				9.2	9.0				.2	0	49.0	5.0	4.6
	c					97.6		.6		2.4				
21	a	8	2.5 oral	+37.9	164	135	16		49	29		29.8	4.6	4.0
	b				7.8	6.4	.75		2.3	1.4	0			
	c					82.3	11.8		37.3	17.7				
28	a	8	2.5 i.p.	+21.2	227	29	9		1	198	13	7.4	3.8	3.6
	b				8.1	.97	.32		.36	7.1				
	c					12.8	31.0		3.5	87.3	41.4			
23	a	7, 8	2.5 i.p.	+20.8	199	30	13			169	13	8.2	2.5	3.5
	b				8.7	1.3	.56			7.4				
	c					15.1	41.3			85.0	56.5			
26	a	8	5.0 i.p.	+28.0	232	1		1		231	25	4.2	4.2	4.1
	b				8.9	.38		.38		8.9				
	c					.44		100.0		99.6	96.2			
37	a	8	10.0 i.p.	+18.3	350					350	37			
	b				9.5					9.5				
	c									100.0	100.0			
24	a	11, 12	5.0 i.p.	+14.7	240	8	5		3	232	22	13.2	3.3	3.4
	b				10.0	.33	.21		.12	9.7				
	c					3.3	62.5		37.5	96.7	91.6			
23	a	11, 12	10.0 i.p.	+11.5	223					223	23			
	b				9.7					9.7				
	c									100.00	100.0			
26	a	15, 16	10.0 i.p.	+34.8	221	207		2		9		37.1	4.7	4.0
	b				8.5	8.0		.1		.4	0			
	c					93.7		.9		4.4				

a = total No. b = per mother rat. c = % of total implants. d = % of live.

fetuses, all present in only 2 litters, out of a total of 240 implantations in this group of 24 rats. The other 22 mother rats had lost their entire litters. The 8 live fetuses found were immature, and 3 also grossly malformed and doubtfully viable (Table I, 7). The 10 mg/kg doses induced 100% litter destruction in 23 rats, while the mothers gained an average of 11.5% of their initial body weight (Table I, 8). Experiments with AZS given to pregnant rats on the 15th and 16th days gave entirely different results. The 10 mg/kg doses had only little effect on the litters, inducing only 5% fetal death, and not destroying a single entire litter in any mother animal (Table I, 9). At autopsy it was evident that neither ovaries nor the corpora lutea of pregnancy nor the placentas were primarily affected by AZS—nor was the pituitary inhibited as the typical lactation changes of the mammary glands indicated. From this one has to conclude that AZS acted directly on the fetus.

Gross malformations of fetuses consisted in hydrocephalus, malformed heads, harelips, skeletal defects, and massive general edema. Stunting consisted in the production of fetuses less than 3.5 cm in length and 3.5 g in weight (See means of normal control litters) (10). Livers and sterna of all mothers were examined histologically and found to be normal.

Placentas. The parasitism of the placentas independent of that of the fetus as first emphasized by Ballantyne(1) was evident in the present experiments. In the experimental rats, approximately one-third to one-half of all the placentas survived the fetuses. Even with complete resorption of the embryos, it was quite usual to find placentas at the site of implantation varying in size from a few millimeters to almost normal size. Three giant placentas were observed in animals where only one or 2 of the fetuses of the litter survived. In 2 instances, the placentas with fetuses weighed 1.7 g each; in the third instance, 1.2 g. The histological structure of the placentas without fetuses varied. Frequently, very small surviving placentas consisted of a central hemorrhagic area surrounded by a rim of giant cells only. When the fetuses were destroyed on the 7th and 8th days of gestation, the surviving placentas failed to show the typical penetration of allantoic vessels into the trophoblast with good labyrinth formation. Surviving fetuses destroyed on the 11th and 12th days of gestation showed abortive labyrinth formation and normal disintegration of the reticularis. In short, changes were noted in the placentas which corresponded to those previously reported by Huggett and Pritchard(6) after crushing of the fetus and hormone administration to the mothers.

Experiments with AZS Given Before Mating. AZS was given to a group of 6-months-old female rats in 2 doses of 10 mg/kg before mating. This amount would have been sufficient to destroy all litters *in utero* any time during the period of implantation to midterm (7th to 12th day). All rats tolerated the drug well and were subsequently mated. Four animals conceived within 2 days, 7 within 3 days and 2 within 4 days, the rest later—a fact illustrating that the drug did not interfere with the ovarian cycle. The rats were found to be pregnant at sacrifice. The weight gain of the pregnant experimental rats was within normal limits; so were the mean number of 9.6 implantations per rat. Of 239 implanted fetuses, 223 or 93.4% were found to be alive. Of these 3 were less than 3.4 cm long, that is, stunted. One fetus was found dead in a litter where 2 other fetuses were completely resorbed. The total number of resorbed fetuses in the 25 experimental rats was 15, or 6.3%, with a standard deviation of $\pm$ 2.4. The mean fetal weight of the experimental animals was 5.3 g, the mean length 4.1 cm, the mean litter weight 47.8 g —all within normal control limits. Most fetal resorptions occurred in litters where the mating had been successful within 3 ovarian cycles from the time of drug administration. The results demonstrated that not sufficient AZS was incorporated in the ova to render them non-viable. However, a small increase in resorbed fetuses over the controls, and also the appearance of a few stunted embryos, occurred (Table II).

TABLE II. Effect of AZS on Rat Litter.

No. of rats		Day of dose	Dose, mg/kg	Mother's wt gain, %	Total implants	Fate of litter: Live	Stunt	Dead	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
25	a	2 ×	2 × 10 AZS		239	223	3	1	15				
	b	before		+38.9	9.6	8.9	.12	.04	.6	0	47.8	5.3	4.1
	c	mating				93.4	1.3	.4	6.3				
12	a	7,8 pro-	5 AZS + pro-		103	5			98	9			
	b	gesterone	gesterone	+21.6	8.6	.4			8.1		6.5	3.5	3.5
	c					4.8			95.0	75			
5	a	7,8 adenine	5 + adenine		53				53				
	b			+11.2	10.3				10.3	100			
	c				100				100				
28	a	7, 8	6MP AZS		246	17			229	26			
	b			+13.8	8.8	.7			8.2				
	c		5.0 1.0			6.9			86.0	93			
26	a	7			248	1	1		247	25	4.0	3.4	4.0
	b		5.0 2.5	+18.9	9.5	.04	.04		9.5				
	c					.4	100		99.5	96.2			
29	a	7, 8			261	2	2		259	28			
	b		5.0 2.5	+14.4	9.0	.1	.1		8.9		6.0	3.0	3.1
	c					.8	100		99.4	96			
21	a	11, 12			192	2	2		190	20			
	b		5.0 5.0	+16.2	9.2	.1	.1		9.1		2.0	1.0	1.0
	c					1.0	100		99.0	95.2			

a = Total No. b = per mother rat. c = % of total implants. d = % of live.

TABLE III. Reproduction Performance of Female Rats after Repeated Complete Litter Destruction with AZS on A—7th and 8th, or B—11th and 12th Days of Gestation.

No. of rats		No. of abortions	Mother's wt gain, %	Uteri total implants	Fetuses			Avg		
					Live	Dead	Resorb.	Litter wt, mg	Single fetus Wt, mg	Single fetus Length, cm
37	a			352	346		.3			
	b	0	+36.1	9.5	9.4	—	.08	44.6	4.7	4.4
	c	control			99.1		.8			
12	a			108	108					
	b	1*	+36.0	9.0	9.0	—	—	58.8	6.5	4.5
	c				100					
9	a			74	7.4					
	b	2*	+38.0	8.2	8.2	—	—	45.7	5.6	4.2
	c				100					
10	a			73	73					
	b	3*	+37.3	7.3	7.3	—	—	48.5	6.2	4.2
	c				100					
10	a			94	82	9	.3			
	b	4*	+38.0	9.4	8.2	.9	.3	50.3	5.4	4.1
	c				87	11 ‡	3.2			
9	a			79	79					
	b	5*	+38.0	8.6	8.6	—	—	57.6	6.0	4.6§
	c				100					
10	a			81	81					
	b	1†	+37.0	8.1	8.1	—	—	52	5.4	4.1
	c				100					
30	a			239	216	18	5			
	b	2†	+35.0	7.9	7.2	.6	.16	48	6.1	4.2
	c				90.4	8.4‡	2.1			
6	a			59	52	3	4			
	b	3†	+36.0	9.8	8.6	.5	.6	52.4	5.8	4.2
	c				88.2	5.8‡	6.8			
10	a			73	73					
	b	4†	+37.0	7.3	7.3	—	—	49	6.7	4.5§
	c				100					

* Litter destruction on 7th and 8th days of gestation with 5 mg/kg.
† " " " 11th and 12th " " " " 10 " .
‡ Percentage of live.
§ Reared and inbred.
a = total. b = per animal. c = % of total.

Effect of progesterone and adenine with AZS on rat litter. In addition to the 5 mg/kg AZS on the 7th and 8th days of gestation a group of animals received on the 7th, 8th and 9th days, one hour before the AZS medication, 10 mg of progesterone intramuscularly. At sacrifice no protective effect of progesterone was noted; 95% of all the fetuses were resorbed, and the 5 survivors small (Table II, 2). An attempt to prevent the action of AZS on the litters by giving large doses of Adenine also failed. Five rats treated with 5 mg/kg AZS on the 7th and 8th days of gestation received 250 mg of adenine sulfate 1 hour before the AZS. At sacrifice all litters were completely resorbed. The kidneys of the mother animals showed the typical "adenine kidneys" previously described (Table II, 3).

AZS in combination with 6 mercaptopurine (6MP) (Table II, 4,5,6,7). 6MP was previously shown to destroy the rat litter in doses not less than 10 mg/kg. In combination with AZS it was more efficiently destroying the entire litters when given as listed on Table II, than either compound alone. All survivors in these groups were stunted and not viable.

Repeated litter destruction with AZS (Table III). After it was shown that AZS

as described would destroy efficiently the entire litters of rats, groups of pregnant animals were exposed to repeated consecutive abortion with the drug. One group was repeatedly aborted at the time of implantation, the other at midterm. The rats were then permitted to become pregnant again and go untreated to term. It was striking to observe that all the aborted rats would not mate until the natural term of their interrupted pregnancy would have arrived (22 days); the rats then ovulated and mated. The offspring of rats thus aborted were normal in all respects, in numbers, size and weight. Gross abnormalities were not seen nor were they detected on x-ray photography of the grown-up litter. Subsequent inbreeding of litters out of mothers thus aborted 4 to 5 times produced again normal offspring in all respects.

Discussion. The present experiments have again demonstrated that 1 or 2 doses of AZS representing only 1/18 of 1/7 of the single LD_{50} for the adult mother rat destroyed the entire litters during the first half of gestation period (7th to 12th day)(7,8). Because of the great differential action between doses lethal to the litter and toxic to the mothers, AZS is an efficient and also safe compound for the purpose of litter destruction. Furthermore, the extended action of the drug covering the time from implantation to midterm is remarkable. Other drugs have failed to have such drastic effects on the litter except for the first 48 hours following implantation(9). The malformations observed in the present experiments corresponded to those previously recorded following administration of this and other antimetabolites (7,8).

Summary. 1. The observation that AZS affects the rat fetus *in utero* is confirmed and greatly extended. 2. AZS acts on the fetus directly—and not on the ovary, placenta or pituitary. 3. AZS efficiently destroys the entire litter of rats in doses of: (a) 5 mg/kg at the time following implantation (7th and 8th days of gestation); (b) 10 mg/kg at midterm (11th and 12th days of gestation); without affecting the mothers adversely. 4. This drug effect is sharply limited in time. 5. Combination of 6MP and AZS was effective in litter destruction in smaller doses than either compound alone. 6. Neither progesterone nor adenine protected the litter against AZS. 7. AZS given before mating did not delay it, nor did it affect the subsequent litter. 8. Rats aborted 4 and 5 consecutive times with AZS showed no accumulative toxicity nor impairment of their fertility. 9. Litters raised from rats aborted 4 or 5 times were normal in all respects and produced normal litters.

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Effect of 6 Diazo 5 Oxo L-Norleucine (DON) on the Rat Litter *in Utero*.* (22848)

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DON—a diazoketone—was first isolated from a streptomycelium and later synthesized. The compound gave promise of tumor inhibition in experimental tumors as well as in human leukemias by inhibiting purine synthesis *de novo,* as reported. DON was first reported by Murphy and Karnofsky to affect the chick and rat embryo adversely, in doses smaller than those of Azaserine (AZS)(4). The present experiments were carried out to determine its effect on the litter of pregnant rats.

Methods. The Long Evans strain of rats was used in the present experiments. The experiments were conducted as previously described in detail(5). Only rats which had one previous satisfactory litter were used. Vaginal smears were done daily, to determine the onset of pregnancy. The compound was given by intraperitoneal injection, dissolved in sterile water, in such concentration that the animals received 1 cc of solution for each 100 g of body weight. Due to the instability of the drug, the watery solution was frozen solid until the time of injection. Any defrosted surplus was discarded. The experimental animals were all sacrificed on the 21st day of gestation. At autopsy the internal organs were inspected, the uteri removed, and contents noted and recorded as described before. The results were listed and compared with the controls (Table I).

Results. The weight changes of the experimental animals corresponded to those of the controls and evidenced their wellbeing. The internal organs of the mother animals were found to be within normal limits—especially the liver, bone marrow and pancreas.

The first group of rats, treated with .05 mg/kg on the 7th and 8th days of gestation (Table I, 2) showed an increased number of fetal resorptions, but no malformations. The second group, treated with a single dose of .3 mg/kg on the 8th day of gestation, responded with 92% of all fetuses resorbed, and 62% of all litters destroyed. Stunting was noted in one fetus (Table I, 3). The same dosage, given at 3-hour intervals, twice on the same day, induced death in all but 1 stunted and malformed fetus, and completely destroyed the litter in all animals but 1 of a group of 21 rats (Table I, 4). The results indicated that if efficient litter destruction was to be obtained, a larger amount of drug had to be given. On raising the dosage to .5 mg/kg given on the 4th and 5th days of gestation, 39% of all the fetuses, but only 12.5% of all litters, were destroyed. Stunting and malformation increased to 10% of the surviving fetuses (Table I, 5). The single doses of .5 mg/kg, given on the 8th day of gestation, resulted in death and resorption of 96% of all the fetuses, but only in 77% total litter destruction. The surviving fetuses were all stunted and malformed (Table I, 6). The dosage of .5 mg/kg given twice on the 7th and 8th days of gestation, or on the 11th and 12th days of gestation, resulted in complete litter destruction without survivors (Table I, 7, 8).

DON and progesterone. Attempts were made to protect the fetuses against the action of DON on the 7th and 8th days of gestation, with 3 injections of 10 mg of progesterone given intramuscularly on the 6th, 7th and 8th days of the gestation period. At sacrifice, 82% of the fetuses were found to be resorbed, and all the surviving embryos were found to be stunted (Table I, 9).

DON and adenine. Another group of rats, treated on the 7th and 8th days of gestation with .5 mg DON, received 1 hour before each injection of the drug 250 mg of adenine sul-

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TABLE I. Effect of DON on Rat Litter *In Utero*.

No. of rats		Day of dose	Dose, mg/kg	Mother's wt gain, %	Total implants	Fate of litter: Live	d	Dead	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
95	a	7,8 p*	Controls		905	891		1	13				
	b		p*	+39.1	9.5	9.4		.01	.14	0	45.7	4.9	4.3
	c					98.5		.11	1.4				
25	a	7, 8			217	163		2	52				
	b		.05	+35	8.7	6.5		.08	2.1	0	37.1	4.5	3.9
	c					75		.9	24				
16	a	8			135	11	1		124	10			
	b		.3	+12.4	8.4	.7	.9		7.7		8.1	4.5	3.9
	c					8.2	9.1		92	62.5			
21	a	8, 8			199	1	1		198	20			
	b		.3, .3	+ 5.8	9.5	.05	.05		9.4		3.1	3.1	3.5
	c					.5	100		99.9	95.4			
24	a	4, 5			172	104	10		68	3			
	b		.5	+18.3	7.2	4.3	4.1		2.9		23.4	4.7	3.7
	c					60.5	9.9		39.5	12.5			
26	a	8			219	7	7		212	20			
	b		.5	+16.6	8.4	.27	.27		8.1		6.0	3.4	3.4
	c					3.2	100		96.8	77			
24	a	7, 8			189				189	24			
	b		.5	+ 7.7	7.8				78				
	c								100	100			
25	a	11,12			184				184	25			
	b		.5	+12.3	7.5	0			7.5				
	c								100	100			
16	a	7, 8			156	27	27		129	8			
	b		.5 + pro*	+17.2	9.7	1.7	1.7		8.0		10.7	3.5	3.4
	c					17.3	100		82.6	50			
7	a	7, 8			62	45			17				
	b		.5 + ade*	+24	8.8	6.4			2.4	0	38	4.3	4.7
	c					72.5			27.4				

* p = placebo; pro = progesterone; ade = adenine.
a = total No. b = per mother rat. c = % of total implants. d = % of live.

fate. At sacrifice, only 20% of all the fetuses were found to be resorbed, and not a single litter was entirely destroyed. The mothers showed typical adenine kidneys(3). The results indicated that adenine antagonized the action of DON. The surviving fetuses were found to be normal (Table I, 10).

Action of DON. At sacrifice, the maternal internal organs were found to be normal. The mammary glands were lactating, the ovaries presented corpora lutea of pregnancy, the livers, bone marrow and pancreas were found to be normal. From this and the survival of numerous placentas to term, one had to conclude that DON, like AZS, acted directly on the fetuses. The malformations observed corresponded to those described for folic acid antagonists and AZS, consisting predominantly of skeletal defects(2).

Placentas. In many instances, the placentas survived the resorbed fetuses. Many uteri were studded with placentas of varying sizes without a single fetus. The largest number of surviving placentas without fetuses was noted in Group 3, Table I, where 75% of all the placentas were found at sacrifice. Group 5 and Group 9 followed, with 44% and 53%. Group 7 and Group 8 in Table I, however, showed only a minimum of 2% of surviving placentas.

Effect of repeated litter destruction with DON on fertility and subsequent offspring of the rat (Table II). After the litter-destroying doses of DON (2 times .5 mg/kg) (Table

TABLE II. Reproductive Performance of Female Rats after Previous 4 Abortions with .5 mg/kg DON on 7 + 8 Day of Gestation.

No. of rats		Mother's wt gain, %	Total implants	Fate of litter		Avg litter wt	Avg single fetus	
				Live	Res.		Wt, g	Length, cm
9	a		68	68				
	b	+25.3	7.5	7.5	0	47.5	6.3	5.0
	c			100				

a = total No. b = per mother rat. c = % of total implants.

I, 7) was determined, it was of interest to study the effect of multiple consecutive litter destruction in the same mother animals. For this reason, a group of mature rats who had previously had normal litters were aborted 4 consecutive times, on the 7th and 8th days of gestation, with DON. Several of the rats died during the experiment from infection and other complications such as pyometra, uterine hemorrhage, and pneumonitis. Delay was noted in re-mating of the female rats following litter destruction. It occurred at the natural end of term, between 23 and 30 days after sperm was found in the vagina, and 14 to 20 days after DON administration. Failure to detect sperm in the vagina of the experimental rats led in 2 instances to the production of litters after only 1 previous abortion, and in 4 instances after 2 previous abortions. The litters of these rats appeared to be normal in all respects. In 7 instances, rats delivered stunted small litters with skeletal malformations in spite of DON administration, during the 3rd period of gestation. This was due to disintegration of DON in the watery solution at room temperature.

After 4 consecutive abortions, the rats were re-mated and permitted to go untreated to term (Table II). The litters appeared to be normal. Neither the ovarian cycle nor fertility was adversely affected by previous DON administration.

Discussion. In comparison to AZS, DON is a less stable compound, especially in solutions with a pH of less than 6.7. It is therefore more difficult to preserve in a watery solution. On the other hand, the compound appears 10 times more active in total litter destruction than AZS(4), while offering a greater margin of safety between toxic doses to the mothers and fatal doses to the litters. Similarly to AZS, DON also permits complete litter destruction from the time of implantation to mid-term(4). Unlike AZS, adenine protected a large number of fetuses against the action of DON, which supports the theory of DON's inhibition of the *de novo* synthesis of purine bases(1).

Summary. DON affects adversely the fetus when given from time of implantation to mid-term, and two doses of .5 mg/kg during this time will result in complete litter destruction. The action of DON appears to be directly on the fetus and not on placenta, ovary or pituitary. Progesterone given prior to DON administration did not protect the litters. Large doses of adenine sulfate given prior to DON administration protected the litters to a large degree. Repeated complete litter destruction in the same animals gave no evidence of cumulative toxicity, nor did DON adversely affect the fertility of the mother rats, or subsequent offspring.

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Effect of 2,4,6, Triamino-"S"-Triazine (TR), 2,4,6 "Tris" (Ethyleneimino)-"S"-Triazine (TEM) and N, N′, N″-Triethylenephosphoramide (TEPA) on Rat Litter *in Utero*.* (22849)

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Previous experiments had shown that some antimetabolites introduced into chemotherapy of cancer have an adverse effect on fetal development when given to the mother rats in the first trimester of pregnancy(7). In the present experiments, TR, TEM and TEPA were given to pregnant rats in order to determine their action on the growing fetus and litter.

Procedure. The experimental procedures employed are mentioned in detail elsewhere (7). *Methods.* Groups of pregnant female rats were given the compounds on the 4th and 5th, or the 7th and 8th, or the 11th and 12th days of gestation, counting the morning of massive sperm findings in the vagina as day 0. The rats were kept in single cages, fed *ad lib.*, the "White Breeding" diet of Evans and Long, and were sacrificed under ether anesthetic on the 21st day of gestation, or the day before the expected littering. Only rats with known breeding performances were used. All animals had one previous litter, were approximately 6 months old, and weighed between 200 and 300 g at the time of mating. Weight curves of the animals were kept. The internal organs were inspected, the sternum and spleen fixed and sectioned. The uteri were removed *in toto,* opened longitudinally, and the placentas and fetuses measured, weighed, recorded, x-rayed and then fixed in 10% formalin, stained and cleared. *Dosage.* According to F. S. Philips(5,6), the 5 LD_{50} for the Wistar rats, for Triazine is approximately 70 mg/kg, for TEM is .3 mg/kg, and for TEPA 5 mg/kg. The 5 LD_{50} was given twice on subsequent days. TR and TEM were injected intraperitoneally, TEPA intramuscularly.

Results with TR. TR administration had only a minor effect on the fetuses or litters of the experimental rats. The number of resorptions in the group treated on the 4th and 5th days of gestation exceeded those of the controls, but is not significant (Table I). The findings demonstrate that TR, itself forming the basic molecule of TEM, is harmless to the rat fetus.

TEM. The compound induced 78% fetal resorption but only 25% total litter destruction

TABLE I. Effect of Triazine on Rat Litter *In Utero*, 70 mg/kg Intraperitoneally.

No. of rats		Day of dose	Mother's wt gain, %	Total implants	Fate of litter				Mothers with all resorb. litters	Avg litter wt	Avg single fetus	
					Live	Dead	Gross malf. d	Res.			Wt, g	Length, cm
21	a	4, 5	+28.2	176	160	1		13				
	b			8.4	7.6	.57		.62	0	41.0	5.0	4.2
	c				91			7.4				
26	a	7, 8	+39.9	221	213	2		6				
	b			8.5	8.2	.07		.23	0	46.5	5.7	4.1
	c				96.4	.9		2.7				
26	a	11, 12	+33.3	230	224			6				
	b			8.8	8.6			.23	0	42.7	4.4	4.1
	c				97.4			2.6				

a = total No. b = per mother rat. c = % of total implants. d = % of live.

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TABLE II. Effect of TEM on Rat Litter *In Utero*.

No. of rats		Day of dose	Dose, mg/kg	Mother's wt gain, %	Total implants	Fate of litter: Live	d Stunt	Dead	d Malf.	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
24	a				202	41	5	2		159	6			
	b	4, 5	.3	+12.5	8.4	1.7	.2	.1		6.6		11.9	4.4	3.6
	c					20.3	2.5	1.0		78.8	25			
26	a				233	49	23	1	1	183	9			
	b	7, 8	.3	+19.0	9.0	1.9	.9	.4	.4	7.0		8.2	4.3	3.5
	c					21.0	47.0	.4	2.0	78.6	34.6			
26	a				245	201	13	4	1	40				
	b	11, 12	.3	+30.4	9.4	7.7	.5	.2	.4	1.5	0	35.2	4.6	3.9
	c					82.5	6.5	1.6	.5	16.3				
18	a				153					153	18			
	b	7, 8	.5	+ 6.9	8.5					8.5				
	c									100	100			
21	a				163	111	66	29	19	23				3.5 live
	b	15, 16	.3 + AZS	+14.0	7.8	5.3	3.1	1.4	.9	1.1	0	22.0	3.3	
	c					68.1	59.5	17.8	13.6	14.1				2.9 dead
14	a				123	52	9	1		71	5			
	b	7, 8	.5 + pro-	+18.3	8.8	3.7	.64			5.0		23.5	4.1	3.6
	c		gesterone			42.2	17.3			57.2	35.7			

a = total No. b = per mother rat. c = % of total implants. d = % of live.

TABLE III. Effect of TEPA on the Rat Litter *In Utero*, 5 mg/kg Intramuscularly.

No. of rats		Day of dose	Mother's wt gain, %	Total implants	Fate of litter				Mothers with all resorb. litters	Litter wt, g	Avg single fetus	
					Live	d Malf. or stunt	Dead	Res.			Wt, g	Length, cm
24	a	4, 5	+18.3	211	36	6		175	10	13.0	5.0	4.1
	b			8.8	1.5	0.24		7.3				
	c				17.1	16.7		82.9	41.7			
19	a	7, 8	+15.9	146	30	7		116	10	13.2	3.9	3.7
	b			7.7	1.6	0.37		6.1				
	c				20.5	23.3		79.5	52.7			
22	a	11, 12	+10.0	191	15	15		176.0	10	4.5	3.1	3.3
	b			8.7	.7	.7		8.0				
	c				7.9	100.0		92.1	45.5			
23	a	11, 12	+ 8.6	209				209	23			
	b	new		9.1				9.1				
	c	sol.						100.0	100.0			
19	a	7, 8	+ 5.9	178				178	19			
	b	new		9.4				9.4				
	c	sol.						100.0	100.0			

a = total No. b = per mother rat. c = % of total implants. d = % of live.

before implantation. The maximum effect of two doses of .3 mg/kg on the litter was found at the time of implantation. 78% of all the fetuses were found dead or resorbed, but only 38.5% of all litters were destroyed (Table II, 2). An increase in dosage from 2 x .3 mg/kg to 2 x .5 mg/kg given at this sensitive period induced 100% litter destruction, while still permitting a weight gain of 6.9% of the mothers (Table II, 4), within the normal range of untreated control rats. At midterm 2 doses of .3 mg/kg failed to destroy a single litter and induced only 16.3% of fetal resorptions. Attempts to destroy the litter *in utero* on the 15th and 16th days of gestation with 2 x .3 mg/kg TEM and 2 x 10 mg/kg of Azaserine (AZS) failed entirely (Table II, 5). Not a single litter of 21 pregnant rats was destroyed, only 17.8% of all fetuses were found dead *in utero*, and only 14% of all embryos resorbed; however, nearly 60% of all living fetuses were found to be stunted or malformed. An attempt was made to protect the litter of rats treated on the 7th and 8th days of gestation with .5 mg/kg TEM, by giving 10 mg progesterone intramuscularly 1 hour before the TEM administration on the 7th and 8th days, as well as further 10 mg on the 9th day. Progesterone permitted 42% of the fetuses to survive and reduced the litter effect to 35%. Stunting was not prevented (Table II, 6). *Stunting.* The smallest live fetus measured 2.6 cm in length, and was observed in an animal treated with .3 mg/kg on the 7th and 8th days of gestation. In the same group, 10 other fetuses measured only 3 cm in length. The group of rats treated with TEM and AZS on the 15th and 16th days of gestation showed 35 fetuses varying in length from 2.5 to 3 cm, the remainder of the stunted embryos varying from 3 to 3.4 cm in length. *Malformations.* The malformations were predominantly cranial defects, harelip, anencephaly, encephalocele, abnormalities of the facial structures, and general edema and anasarca of the body.

TEPA. The results are listed in Table III, and indicate that this compound was most active in litter destruction from the 7th and 8th to the 11th and 12th days of gestation. The first series of experiments (Table III, 1, 2, 3) was carried out with the compound suspended in sesame oil for 22 months. However, when the experiments were repeated with a fresh compound (Table III, 4, 5) 100% litter destruction was obtained. Animals treated on the 11th and 12th days had a tendency to bleed from the uterus. Several died spontaneously on the 20th day of gestation. The effect on the litter and fetuses be-

fore implantation exceeded that of TEM with 41% of total litter destruction and 83% of all fetuses resorbed. *Stunting.* Of 15 surviving fetuses of the group of rats treated on the 11th and 12th days of gestation, 6 measured only 3.3 cm and the remainder 3.4 cm. Four fetuses of the group treated at the time of implantation varied in size from 2.5 to 3 cm. Four others were less than 3.5 cm. In the group of rats treated on the 4th and 5th days of implantation, one measured only 2.3 cm and one other only 3.4 cm in length. *Malformations.* Two fetuses with malformations of the cranium, one anencephalus with ventral hernia, and one fetus with a facial defect, were observed.

Mother animals. The appetite of the mother rats was not impaired during or after drug administration. Weight loss or diarrhea did not occur. However, a number of rats were lost from uterine hemorrhages on the 20th day of gestation, especially in the group of rats treated with TEPA on the 11th and 12th days of gestation. *Microscopic Examination.* Bone marrow and spleens of rats treated with TR were found to be normal at sacrifice. The bone marrow and spleen of the rats treated with TEM were found to be normal with 2 exceptions showing a 30% depletion of the total marrow cellularity. The histological findings of bone marrow and spleens were in most cases those of an actively regenerating erythro- and myelopoiesis—the animals having completely recovered from the marrow injury. The groups of rats treated with TEPA still showed at sacrifice definite evidence of marrow depression, ranging from 15% to 35%, the normally active cellular marrow being replaced by hemorrhages and fat.

Placentas. The average discoid placenta of our control fetuses weighed .8 g and measured .9 x .4 cm in diameter. The placentas of surviving fetuses of rats treated with TEM were uniformly smaller than those of the controls, but the fetuses were of normal size and weight. The placentas of the group of rats listed in Groups 2 and 3 of Table II weighed only .4 g, and measured only .4 x .2 cm in diameter. Some of the placentas of group 4 in Table II weighed only .6 g and measured .6 x .3 cm in diameter.

% of surviving placentas without fetuses:

Drug	Days of drug administration			
	4th & 5th	7th & 8th	11th & 12th	15th & 16th
TR	100	100		
TEM	14	75	40	94
TEPA	14	5.1	28	94
TEM & AZS				74

The generally decreased size of placentas in the TEM treated rats and the protective action of progesterone on the fetuses against the otherwise lethal doses of TEM suggest strongly a TEM effect on the placenta. Rats treated with TEPA had larger placentas, corresponding to those found in the controls. The percentage of placentas surviving the fetuses also differed from the TEM groups.

Discussion. Nitrogen mustard was previously shown to have a very deadly effect on the 10-day-old rat embryos(2,4). TEM and TEPA are compounds very similar in action to the nitrogen mustards(5). Their effect on the bone marrow, lymphoid system, intestinal and testicular epithelium has been described (5,6). The compounds were also shown to induce chromosomal abnormalities producing bridging and linkage of chromosomes in mitosis(1). TEM and TEPA are used in palliative chemotherapy and Hodgkin's disease and lymphoma in men(3,6). It was therefore of great interest to study the effect of TEM and of TEPA on the rat litter *in utero.* The results have shown that TEPA in 2 doses of the 5 LD_{50} would destroy with greater certainty the entire litter of rats at the time of implantation, but also that by comparison to TEM, TEPA induces a more lasting bone marrow depletion and intoxication of the rat. This might be due to a slower rate of resorption from the site of injection. TEM appeared to inhibit the growth of the placenta to a minor degree. Its effect on the fetus was surprisingly counteracted by simultaneous doses of progesterone. The findings suggest that TEM acts therefore not only on the fetus but also on the placenta. The pituitaries of the present rats treated were not affected, judged on the secretory activity of

the mammary glands of the rats at sacrifice.

Summary. 1. The effect of TR, TEM and TEPA on rat fetus and litter *in utero* was studied with 2 doses of the 5 LD_{50} on 4th and 5th, or 7th and 8th, or 11th and 12th days of gestation. 2. TR had no effect on the rat litter and can be regarded as a control series to TEM. 3. TEM had its maximum effect at the time of implantation, but failed to destroy the entire litters. An increase in dosage from .3 mg/kg to .5 mg/kg given at the time of implantation led to 100% litter destruction. 4. Progesterone administration protected 40% of the fetuses against otherwise lethal doses of TEM. 5. Rats treated with TEM displayed uniformly smaller placentas than normal. 6. TEM in combination with AZS given on the 15th and 16th days of gestation led to much stunting of the fetuses, to 32% of fetal death, but in no instance to complete litter destruction. 7. Freshly prepared TEPA destroyed all litters when given on the 7th and 8th, or 11th and 12th days of gestation. 8. The malformations observed with TEM and TEPA consisted of cranial defects and abnormalities of facial structures. 9. TEM and TEPA in doses used affected adversely the bone marrow and lymphoid system of the mother animals.

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Effect of 2-6 Diaminopurine (2-6 DP): 6 Chlorpurine (ClP) and Thioguanine (ThG) on Rat Litter *in Utero.** (22850)

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2-6-DP, CIP and ThG were synthesized as members of a larger group of purine analogues(1,4). Their biological activity was extensively investigated in microorganisms (6), as well as in the embryo of *Rana pipiens* (2), adult mice, and rats(8) and experimental tumors(4). Following the study of 6 Mercaptopurine (6-MP) which showed that the dosage of 10 mg/kg and more would destroy most of the fetuses of the pregnant rat *in utero* at the time of implantation(9), the 3 compounds were investigated for their action on the rat litter.

Procedure. Long Evans strain of rats was used exclusively in the present experiments. All rats were 6 months old, had one previous satisfactory litter, and weighed 200 g or more. For details of reproductive performance of the strain of rats used, diet and technics employed, see previous publication(9). The onset of gestation was determined by finding of vaginal sperm. All animals were injected intraperitoneally strictly on a mg/kg basis. Experimental animals and controls were sacrificed on the 21st day of gestation, the day before littering, to avoid loss of fetuses or placentas. The internal organs of the mother animals were sectioned, the uteri removed *in toto,* and their contents noted. Placenta and fetuses were counted, measured and weighed, and many of the fetuses sectioned or cleared.

Toxicity data. Single and 5 LD_{50} for each compound is listed as given by Philips for the Wistar rat(8).

Rat toxicity in mg/kg:

	2-6-DP	ClP	ThG
Single LD_{50}	100	500	300
5 LD_{50}	50	100	10

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TABLE I. Effect of 2,6-Diaminopurine on Rat Litter *In Utero*, 50 mg/kg IP.

No. of rats		Day of dose	Mother's wt gain, %	Total implants	Fate of litter: Live	Malf. or stunt[d]	Dead	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
21	a	4, 5	+13.9	167	42			125	12	21.8	4.7	4.6
	b			8.0	2.0			6.0				
	c				25.2			74.8	57.2			
23	a	7, 8	+25.1	167	143	17	3	21		29.8	4.8	3.9
	b			7.3	6.23	0.74	0.13	0.91	0			
	c				85.6	11.9	1.8	12.6				
20	a	7, 8 +	+32.3	165	148	5		17		39.5	5.3	4.1
	b	lactate		8.25	7.4	0.25		0.85	0			
	c				89.7	3.8		10.3				
26	a	11, 12	+26.3	220	208		2	10		40.0	5.0	3.8
	b			8.5	8.0		0.1	0.4	0			
	c				94.6		0.9	4.5				

a = total No. b = per mother rat. c = % of total implants. d = % of live.

Results. To avoid toxicity to the mother animals, the compounds were injected intraperitoneally in 2 doses of the 5 LD_{50}, only. Groups of pregnant rats were injected on the 4th and 5th, or the 7th and 8th, or the 11th and 12th days of gestation to determine the effects of the drugs before implantation, at the time of implantation, and at midterm. 2-6-DP was prepared with lactate to render the compound more water-soluble and easier to inject. 2-6-DP had a peak of action when given before implantation. 75% of all fetuses were resorbed, and 57% of all litters completely destroyed (Table I). On the 7th and 8th days of gestation, the drug failed to destroy a single litter, but induced malformation and stunting in 12%, and resorption of 12% of all fetuses (Table I, 2). In a second series, treated in the same time period, 2-6-DP was given without lactate. Now only 4% of the fetuses were found to be stunted, and only 10% resorbed. At midterm 2-6-DP had statistically no effect on the litter (Table I, 4). ClP when given before implantation induced 32% of complete litter destruction, with 70% resorption of all fetuses (Table II). Following implantation, it destroyed all the litters (Table II, 2). At midterm only 10% of the fetuses were resorbed, and not a single litter completely destroyed. However, 53% of the

TABLE II. Effect of 6-Chloropurine on Rat Litter *In Utero*, 100 mg/kg IP.

No. of rats		Day of dose	Mother's wt gain, %	Total implants	Fate of litter: Live	Malf. or stunt[d]	Dead	Res.	Mothers with all resorb. litters	Avg litter wt	Avg single fetus: Wt, g	Length, cm
25	a	4, 5	+21.9	247	76	8		171	8	20.9	4.7	3.9
	b			9.9	3.0	0.32		6.9				
	c				30.4	10.5		69.6	32.0			
24	a	7, 8	+ 3.5	242				242	24			
	b			10.1				10.1				
	c							100.0	100.0			
25	a	11, 12	+23.3	203	174	93	7	22	0	23.9	3.4	3.4
	b			8.1	7.0	3.7	0.3	0.8				
	c				85.8	53.2	3.4	10.8	0			
9	a	7, 8 +	+ 1.3	94	0			94	9			
	b	proges-		8.5				8.5				
	c	terone						100	100			

a = total No. b = per mother rat. c = % of total implants. d = % of live.

TABLE III. Effect of Thioguanine on Rat Litter *In Utero*, 10 mg/kg IP.

No. of rats		Day of dose	Mother's wt gain, %	Total implants	Fate of litter				Mothers with all resorb. litters	Avg litter wt	Avg single fetus	
					Live	Malf. or stunt	Dead	Res.			Wt, g	Length, cm
20	a	4, 5	+18.8	142	31	6	4	107	6	13.7	4.2	5.3 live
	b			7.1	1.6	0.3	0.2	5.3		5.0	3.1	2.5 dead
	c				21.8	19.3	2.8	75.4	10.3			
24	a	7, 8	+ 5.2	202				202	24			
	b			8.4				8.4				
	c							100.0	100.0			
23	a	11, 12	+17.2	184	46	17	25	113	10	14.1	2.9	3.2 dead & live
	b			8.0	2.0	0.7	1.1	4.9				
	c				25.0	37.0	13.6	61.4	43.5			
6	a	7, 8 +	+ 5.6	50	9			41	5			
	b	proges-		8.3	1.5			6.8		47.0	4.2	5.2
	c	terone			18.0			82.0	83.5			

a = total No. b = per mother rat. c = % of total implants. d = % of live.

surviving fetuses were stunted or malformed (Table II, 3). Three doses of 10 mg progesterone given intramuscularly on the 7th, 8th and 9th days of gestation before the ClP administration (on the 7th and 8th days only) failed to protect the litters (Table II, 4). *ThG,* when given before implantation, destroyed 75% of all fetuses, but only 10% of all the litters. Stunting was seen in 19% of the survivors (Table III, 1). At the time of implantation, ThG destroyed all litters completely (Table III, 2). At midterm, 61% of all the fetuses were resorbed, and still 43% of all litters destroyed. Thirteen per cent of all the fetuses were found dead, and 37% of the survivors stunted or malformed, indicating the great toxicity of the drug to the fetuses (Table III, 3). Three doses of 10 mg progesterone given intramuscularly on the 7th and 8th and 9th days of gestation, before the doses of ThG on the 7th and 8th days, did not protect the fetuses (Table III, 4).

Action of the drugs. None of the drugs prevented decidua formation or implantation, as the histological studies of the uteri showed. The drugs had little effect on the placentas, as many surviving organs demonstrated. The maternal ovaries carried intact corpora lutea of pregnancy. The mammary glands of most of the animals treated at the time of the 7th implantation or midterm showed glandular proliferation and early lactation, as seen in pregnancy—*ruling out pituitary inhibition by the drugs.* From this, one *must* conclude that the drugs acted primarily on the fetuses. The maternal organs, especially bone marrow, intestinal tract and lymphoid system, were found to be *intact* at the time of sacrifice in the mother rats. If lesions had occurred with drug therapy, the animals had recovered at the time of sacrifice.

Placentas. In many instances the placentas survived the fetuses. A number of uteri containing only placentas of varying sizes, but no fetuses, were noted. Remarkable was the absence of surviving placentas in the groups treated on the 7th and 8th days of gestation with ClP and ThG. Giant placentas, twice normal size, connected with normal size fetuses, were noted 4 times in uteri where the remainder of the litter was destroyed. The percentage of surviving placentas without fetuses in each experimental group of rats is listed below.

% of surviving placentas without fetuses:

Drug	Days of drug administration		
	4th & 5th	7th & 8th	11th & 12th
2-6-DP	12	35	25
6 ClP	60		5.5
ThG	13		72

The *malformations* noted with all 3 compounds consisted of general edema and anasarca; general stunting of the skeleton; cranial defects, with and without hydrocephalus; ventral hernia; situs inversus; incom-

plete development of fore and hind legs.

Weight changes of mother animals. The weight changes of the experimental animals corresponded to those found in control animals, and indicated that no severe intoxication of the experimental animals occurred.

Discussion. The 3 compounds listed in the present experiment differ in their action on the adult mouse and rat tissue. 2-6-DP affects bone marrow, intestinal epithelium and to some degree the lymphoid system. ClP acts on bone marrow and intestinal epithelium; while ThG affects in corresponding doses primarily the bone marrow only(8).

2-6-DP was introduced into experimental chemotherapy of the leukemias(3) and a preliminary trial with the 2 other compounds was carried out(7). For this reason, knowledge of their action on the fetus is important. The findings confirm the studies of Bieber and others on the embryo of *Rana pipiens*(2). By comparison to 6 Mercaptopurine, ClP and ThG match its effects in total litter destruction at the time of implantation; ThG exceeds the action of 6 Mercaptopurine on the rat litter, especially at midterm. All 3 compounds investigated here were more effective on the 4th and 5th days of gestation than 6 Mercaptopurine(9).

Summary. 1. 2-6-DP, ClP and ThG were given to groups of pregnant rats in 2 doses of 5 LD_{50}, not toxic to the mother animals. The compounds were given on the 4th and 5th, or on the 7th and 8th, or on the 11th and 12th days of gestation. 2. 2-6-DP showed a peak of action on the fetuses and litters when given before implantation, destroying 75% of all the fetuses, but only 57% of all the litters. 3. ClP showed a peak of action on the 7th and 8th days, destroying all fetuses and litters. Treatment on days 4 and 5 of gestation destroyed 69% of the fetuses, but only 32% of all litters. At midterm, no litter was completely destroyed, and only 10% of all the fetuses were resorbed. However 50% of all survivors were stunted and malformed. 4. ThG was the most toxic compound to the fetuses. At the time of implantation it destroyed all fetuses. On days 4 and 5 of gestation, it led to resorption of 75% of the fetuses, but only to 10% complete litter destruction. At midterm it still destroyed 61% of all fetuses, and 43% of all litters, while stunting 37% of all survivors. 5. The lethal action of ClP and ThG on the rat litter at implantation time could not be prevented by progesterone administration.

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Alterations in Rat Serum Proteins in Folic Acid and Vit. B_{12} Deficiency.* (22851)

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The known participation of folic acid (PGA) and vit. B_{12} in various single carbon addition reactions implicates these vitamins in the metabolism of glycine, serine, threonine, tryptophane, histidine, and possibly other amino acids and, therefore, in general protein metabolism. This is also inferable from the demonstration that, in a vit. B_{12} deficiency, there is impairment in carbohydrate and lipid metabolism(1) and from the fact that optimal utilization of amino acids depends upon caloric adequacy. It was of interest therefore to study the influence of a deficiency of PGA and vit. B_{12} on the electrophoretic pattern of serum proteins.

Materials and methods. Young male rats (Wistar strain), 50 g in weight were reared on a purified ration consisting of (in g per 100 g of diet): vitamin-free casein, 10; iodinated casein ('Protomone': Cerophyl Laboratories, Kansas City, Mo.), 0.15; sesame oil, 6; shark liver oil, 2; salt mixture (U.S.P. No. 2), 4; sucrose, 9.85; and maize starch, 68; with the following vitamin additions (in mg/kg of diet): thiamine hydrochloride, 6; pyridoxine hydrochloride, 6; calcium pantothenate, 20; riboflavin, 10; biotin, 1; α-tocopherol, 50; nicotinic acid, 30; choline chloride, 500; inositol, 500; and vit. K (Menadione, Merck), 10. The vitamin additions provided in this basal diet were considered adequate for the hyperthyroid condition. At the end of 4 weeks, the animals were divided into 2 groups, one of which continued to receive the deficient diet modified by the withdrawal of iodinated casein and substitution by 2% succinyl sulphathiazole. The second group was fed on the modified diet with additions of PGA and vit. B_{12} at 5 mg and 50 μg respectively per kg of diet. The rats were sacrificed after a further period of 5 weeks and blood was collected from the inferior vena cava. Livers were quickly excised and chilled in cracked ice.

Serum vit. B_{12} was determined by the method as described by Ross(2) using *Euglena gracilis*. Liver vit. B_{12} was determined after liberation from the tissue by overnight incubation under toluene with papain (B.D. H., 25 mg/g of liver) in 0.1 M acetate buffer of pH 4.6; the samples were then autoclaved, homogenized, neutralized and made to volume. Assays were made with *Lactobacillus leichmannii* (ATCC 7830) by a turbidimetric adaptation of the U.S.P. method(3). Liver folic acid was determined after autolysis in 0.2 M phosphate buffer of pH 7.6 using *Streptococcus faecalis R* (ATCC 8043) and the assay medium of Mitbander and Sreenivasan (4).

Electrophoresis of the separated serum was carried out on Whatman No. 3 paper strips 5 x 34 cm in a horizontal, open, strip-type cell (5) with barbital buffer of pH 8.6 and ionic

TABLE I. Blood and Liver Picture in Folic Acid and Vit. B_{12} Deficiency (Averages of 4 Determinations).

Group	Leucocytes/mm^3 ($\times$ 10,000)	Hemoglobin, %	Erythrocytes/mm^3 ($\times$ million)	Liver PGA, μg/g	Liver B_{12}, mμg/g	Serum B_{12}, $\mu\mu$g/ml
Casein (10%) diet without PGA and vit. B_{12}	.47	11.6	3.05	1.8	35.4	170
Casein (10%) diet with PGA and vit. B_{12}	.76	14.6	6.25	6.3	100.8	850

* This work was supported by grant from Williams-Waterman Fund of Research Corporation, N. Y. City.

TABLE II. Fractionation of Rat Serum Proteins in Folic Acid and Vit. B_{12} Deficiency. 7 rats per series.

Group	Total serum proteins	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
	g/100 ml serum						% of total proteins				
Stock diet (20% proteins)	6.93 ± .11	2.79 ± .09	1.34 ± .07	.38 ± .10	1.11 ± .04	1.27 ± .11	40.3 ± 1.4	19.4 ± 1.1	5.5 ± 1.5	16.5 ± .6	18.3 ± 1.7
Casein (10%) diet with PGA and vit. B_{12}	5.35 ± .31	2.13 ± .05	.91 ± .07	.36 ± .07	.97 ± .08	.97 ± .10	39.8 ± .9	17.1 ± 1.4	6.8 ± 1.3	18.1 ± 1.5	18.2 ± 2.0
Casein (10%) diet without PGA and vit. B_{12}	4.16 ± .40	1.38 ± .15	.53 ± .10	.37 ± .05	1.12 ± .14	.76 ± .09	33.1 ± 3.7	12.8 ± 2.5	8.8 ± 1.4	26.9 ± 3.3	18.4 ± 2.2

strength 0.075. Separation was effected at a constant current of 2.5 mA/strip for 16 hours at room temperature (28°C). The subsequent procedure employed was essentially that described by Jencks *et al.*(6). The strips were scanned after immersion in hot liquid paraffin, draining for two hours and blotting off excess paraffin. An 'EEL' Scanner (Evans Electroselenium Ltd., Harlow, Essex) based on the device by Latner *et al.*(7) was used. Total protein was determined by the biuret method(8). Patterns were also obtained for sera from normal adult rats maintained on the laboratory stock diet (20% protein) consisting of (in g/100 g of diet): whole wheat flour, 60; whole milk powder, 10; defatted peanut meal, 11; brewer's yeast, 4; wheat bran, 4; dried fish meal, 3; sesame oil, 4; sodium chloride, 2; and calcium carbonate 2.

Results. In Table I are given the results of blood and liver analyses for the normal and deficient groups of animals at the time when they were sacrificed for fractionation of serum proteins. The latter results are summarized in Table II. Typical profiles for each group are also reproduced in Fig. 1.

It is seen (Table II, Groups 1 and 2) that protein level in the diet alters chiefly the serum concentrations of albumin and α_1-globulin, and, to a less extent, of γ-globulin; their proportions to the total serum protein remain unchanged. In a deficiency of PGA and vit. B_{12} (Group 3), there is a significant reduction in total serum proteins and in serum albumin, α_1-globulin and γ-globulin, although the percentage of total protein as γ-globulin is the same.

Discussion. It has been reported that vit. B_{12} in liver tissue is loosely bound to a protein fraction resembling serum β-globulin in electrophoretic mobility, and that normally, serum vit. B_{12} bound to β-globulin is assayable as free vitamin while that bound to α-globulin is unavailable to assay organisms except after protein denaturation by heating (9). The observed high proportion of β-globulin in group 3 (Table II and Pattern 3) may point to an increased concentration of free vit. B_{12} in the deficient condition. It

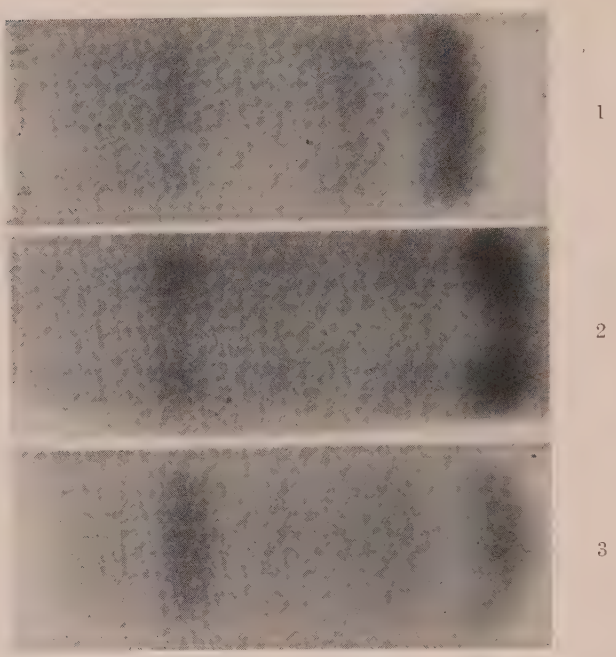

FIG. 1. Alterations in electrophoretic pattern in PGA and vit. B_{12} deficiency. The bands reading from the starting line are, in order γ-globulin, β-globulin, α_2-globulin, α_1-globulin and albumin. There is a decrease in serum levels of albumin, α_1-globulin and γ-globulin with rise in β-globulin level in the deficient animals (Pattern 3). Vit. B_{12} and folic acid supplementation brings these levels to normal (Pattern 2). The serum picture of rats maintained on the stock diet (Pattern 1) has been taken as standard for comparison.

would seem that in a vit. B_{12} deficiency there is increased mobilization of this fraction from the liver to the serum. The increased serum β-globulin observed in the choline-deficient rat(10) may perhaps be similarly explained as due to a greater channelling of vit. B_{12} in this condition. In the present work, the effects of deficiencies of both folic acid and vit. B_{12} on serum proteins were studied in view of their well known metabolic inter-relationships. Experiments are in progress to ascertain their effects singly.

Summary. 1. In a deficiency of vit. B_{12} and folic acid in rats, there is a significant reduction in total serum proteins and in serum albumin, α_1-globulin and γ-globulin. An increase in the proportion of β-globulin is probably indicative of higher serum level of free vit. B_{12} in the deficient state. 2. Dietary protein level influences serum concentrations of albumin, α_1-globulin and, to a lesser extent, γ-globulin.

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Browning in Phosphatide-Containing Fat Emulsions.* (22852)

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Soybean phosphatides are used as primary emulsifier in many fat emulsion formulations developed for intravenous alimentation. These emulsions, in widespread clinical testing, have produced adverse physiological effects(1) of considerable concern. One of the most obvious possible faults of these emulsions is formation of brownish material, mostly colloidal in nature, some of which occasionally settles after several months of storage. This material should be distinguished from filterable foreign material which may enter inadvertently during homogenization process. Various emulsifiers have been investigated in this laboratory, and the brown material has been observed only in those emulsions containing both dextrose and phosphatides. Since nitrogenous systems undergo a browning reaction in presence of glucose, phosphatides present in fat emulsions may be reacting with glucose to form brown material. The browning which occurs in sugar solutions was described by Maillard(2), as reaction between an amino acid and a reducing sugar. Recently, Schroeder *et al.*(3,4) found that the Maillard reaction takes place only in the dry state or at alkaline pH values in aqueous solutions, since only under these conditions is there a decrease in free amino groups. These same investigators found that another browning reaction in acidic solutions occurs without a decrease in concentration of free amino groups but is accompanied by a decrease in aldehyde groups.

The present investigation was undertaken to determine if the nitrogenous groups found in soybean phosphatides were involved in the formation of the brown material. The technic employed to characterize this brown material was paper chromatography.

Materials. A sample of crude, unbleached soybean phosphatides, obtained from commercial source, was freed of oil by repeated precipitation from acetone and of other impurities by filtration through Hyflo Supercel. Another batch of soybean phosphatides, fractionated in a similar manner by a pharmaceutical company for use in emulsions for clinical testing, was compared with those fractionated in this laboratory. The yeast lecithin was prepared by Dr. D. J. Hanahan(5). Choline chloride and animal phosphatides were obtained from Nutritional Biochemical Corp.‡ Dextrose solutions were prepared with Baker's analytical grade dextrose or were 5% dextrose solutions from the Cutter Laboratories.‡

Methods. Chromatograms were obtained by the method of Hanahan(6) and by procedure similar to that used by Dieckert. In both methods ascending technic was used. Ether solutions as well as water slurries of the various substances were employed. The ninhydrin reagent in buffered methyl cellosolve used by Moore and Stein(7) for determination of amino acids and later by Lea and Rhodes(8) for determination of unhydrolyzed phospholipids, was applied to estimation of amino groups present in dextrose solutions containing phosphatides and choline chloride before and after autoclaving. Autoclaving of various materials was at 121°C for 15 minutes at 15 psi. In the Hanahan method, the solvent was a solution of n-propanol, acetic acid, and water (8:1:1). The paper strips were Whatman's No. 1. In the procedure similar to the Dieckert method, the

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‡ Trade names have been used only for the purpose of identifying equipment or materials actually used in conducting the work, and such use does not imply endorsement or recommendation by the U. S. Dept. of Agric. over other firms or similar products not mentioned.

solvent was a solution of absolute methanol and absolute diethyl ether (1:1 by volume). Glass fiber paper was obtained from H. Reeve Angel and Co.‡ The glass fiber strips impregnated with silicic acid were prepared as follows: 100 g silicic acid were dissolved in 100 ml saturated KOH, filtered and made up to 600 ml with water. Strips were immersed in the resultant potassium silicate for 2 minutes, excess silicate removed, and then the strips were immersed in concentrated HCl for 2 minutes. The strips were washed free of chloride ions with water, then washed twice in methanol and twice in ethyl ether, and allowed to dry in air. All strips were equilibrated for half an hour in vapor of the chromatographic chamber before being lowered into the solvent. Usually, about 2 hours were required for the solvent to advance to the top of a 35 cm strip. Reproducible results were obtained with 10, 20, and 50 μl samples of a 1% solution of various phosphatides. In most instances 20 μl samples were used. Strips were dried in air and then stained for choline(9,10), phosphate esters (11), unsaturated groups(12), primary and secondary amino groups(7), sugars(13), and long chain fatty acid groups by use of saturated solution of Oil Red O, a fat soluble dye, in 60% ethanol.

Results. Extent of ninhydrin reactions was determined quantitatively on 0.04% dispersions of soybean phophatides in water and in 5% dextrose solutions, at various pH values before and after autoclaving. Colorimetric measurements were made at 570 mμ with Beckman Spectrophotometer, Model B. These data, Table I, show that at acid pH values there was a definite increase in ninhydrin color, and therefore in the primary and secondary amino groups, with phosphatide dispersions after autoclaving. A type of browning other than the Maillard type is indicated. In that phosphatide dispersion which was alkaline prior to autoclaving, there was no significant increase in optical density. Evidently, another type of browning not resulting in increase in primary or secondary amino groups occurred.

The same ninhydrin procedure was used to measure changes in optical density after autoclaving of solutions of dextrose alone, 1% solutions of choline chloride in water and in dextrose, and 1% dispersions of pure yeast lecithin in water and in dextrose. Changes in optical density on autoclaving at different initial pH values were also observed. These changes are also given in Table I.

TABLE I. Ninhydrin Reactions.

Sample	pH changes on autoclaving	Increase in optical density on autoclaving*
Soybean phosphatides		
In water	5.98–5.68	1.375
In 5% dextrose	5.93–5.28	1.325
Idem, pH 4	4.00–4.52	1.125
" " 8	8.00–5.50	.100
Yeast lecithin (Hanahan)		
In water	6.90–6.82	.040
In 5% dextrose	6.98–5.42	.146
Idem, pH 4	4.05–4.00	.108
" " 8	8.12–5.25	.098
Choline chloride		
In water	5.93–6.10	–.017
In 5% dextrose	5.79–4.27	.028
Idem, pH 4	4.02–4.78	.081
" " 8	7.99–4.52	.031
5% dextrose sol.		
Unmodified	6.50–4.45	.019
pH 4	3.92–3.86	.040
" 8	7.99–4.32	.031

* 570 mμ.

A chromatographic study of the browning reaction products was initiated to determine whether cephalin and lecithin both entered into the observed browning reaction. The chromatographic technics of Hanahan and of Dieckert were compared. The data are summarized in Table II. Glass fiber strips impregnated with silicic acid were used to chromatograph various phosphatides, brown material isolated from emulsions containing soybean phosphatides, and choline chloride. A 1% dispersion of various phosphatides and of choline chloride in 5% dextrose solution and intimate mixtures of "dry" phosphatides or choline chloride and dextrose(1:5 by weight) were prepared. A comparison of the chromatograms of these substances before and after autoclaving was made. The R_f values are given in Table III.

TABLE II. R_f Values Obtained by Chromatography on Cellulose and Glass Fiber Strips.

	R_f values							
	Reineckate positive & phosphomolybdic acid positive spots		Ninhydrin positive spots		Positive spots for unsaturation		Positive spots for Lipids	Phosphate esters
Materials	Cellulose	Glass fiber	Cellulose	Glass fiber	Cellulose	Glass fiber	Cellulose	Glass fiber
Soybean phosphatides (supplied by pharmaceutical co.)	.74	.27	.06 .65	.02 .81	.72	.26 .78	.77	.04 .30
Soybean phosphatides (fractionated in this laboratory)	.74	.23	.07 .65	.05 .81	.72	.23 .81	—	.05 .31
Yeast lecithin (supplied by D. J. Hanahan)	.68	.07 .31	—	—	.80	.07 .31	—	.04 .31

Discussion. The method employing glass fiber impregnated with silicic acid required only 2 hours for solvent to travel as far as it did in 16 hours by using cellulose strips. Cephalin and lecithin travel at greatly different speeds on glass fiber whereas on paper strips, their R_f values are not too different. Sensitivity determinations for choline with both Reineckate(9) and phosphomolybdic acid(10) tests were made on both cellulose and glass fiber strips. With the phosphomolybdate test, 100 μg of phosphatides were required to give a good positive test for choline on glass fiber strips, but 200 μg were required on cellulose strips. When the Reineckate test for choline was used, 400 μg of phosphatides were required for a good positive test for choline on glass fiber strips, whereas only 300 μg were required on cellulose strips.

The R_f values obtained with ether solutions of animal phosphatides or pure yeast lecithin were identical with those obtained with water dispersions. The R_f values for soybean phosphatides in water were unlike those in ether, but like those in dextrose solution because soybean phosphatides contained naturally occurring sugars, whereas animal phosphatide and yeast lecithin did not. In the presence of dextrose solutions, yeast lecithin formed a compound which gave a positive choline spot with an R_f value of 0.55. The choline chloride, animal lecithin fraction, and soybean lecithin fraction formed compounds with dextrose which gave choline positive spots with R_f values of 0.43-0.44. In all cases except with choline chloride, positive phosphate ester spots were also obtained at these new R_f values, indicating that the reaction products with dextrose still contained the phosphate ester group. There is evidence of reaction between choline-containing phosphatide compounds and dextrose to produce a type of compound containing choline. This compound is produced whether or not the lecithin fraction is autoclaved, and in the mixture of phosphatides, regardless of whether or not water is present during autoclaving process. The resultant complex has an R_f value greater than that of the original choline-containing compound. It is evident from Table III that choline chloride and pure yeast lecithin formed other reaction products with dextrose. The ninhydrin test was strongly positive and gave R_f values for products of choline chloride and lecithin of 0.72 and 0.91, respectively. The reaction product between pure yeast lecithin and dextrose also gave a strong positive test for phosphate ester group at an R_f value of 0.90.

In those phosphatides containing cephalins, a choline positive spot with R_f value from 0.72 to 0.80 was obtained when dextrose and water were present. This choline positive spot was not obtained with choline chloride or yeast lecithin in presence of dextrose, and therefore was probably the result of interaction of primary and secondary amino groups with dextrose to produce tertiary amino complexes.

TABLE III. Summary of Chromatographic Data.

Materials	R_f values: Reineckate positive and phosphomolybdic acid positive spots (lecithin fraction)				Ninhydrin positive spots (cephalin fraction)		
Pure yeast lecithin							
Ether solution	.07	.31					
Water dispersion	.07	.30					
Dispersion in 5% dextrose solution	.05	.29	.55			.61	
Idem, after autoclaving	.09	.31	.55				.91
Water dispersion of product autoclaved dry with dextrose	.05	.28					.92
Choline chloride							
Dextrose solution		.24	.45			.71	
Autoclaved dextrose solution			.44			.72	
Water dispersion of product autoclaved dry with dextrose		.24				.73	
Soybean phosphatides*							
Ether solution		.25			.05		.81
Water dispersion			.44	.80†	.04	.74	
Autoclaved water dispersion			.43	.77	.04	.74	
Dispersion in 5% dextrose solution		.28	.44	.77	.04		.80
Autoclaved dispersion in 5% dextrose		.29†	.44	.75	.04	.75	
Water dispersion of product autoclaved dry with dextrose			.50			.72	
Water dispersion of autoclaved dry phosphatides		.28	.56		.04	.73	
Animal phosphatides‡							
Ether solution	.03	.26			.05	.74	.82
Water dispersion	.03	.26			.04	.73	.82
Autoclaved water dispersion	.03	.26			.04	.73	.82
Dispersion in 5% dextrose solution	.05			.72	.06		.79
Autoclaved dispersion in 5% solution	.04			.73	.04	.70	
Water dispersion of product autoclaved dry with dextrose		.29*	.44	.75	.04	.75	
Brown material isolated from emulsions							
Water dispersion	.03	.26	.52	.80	.03	.76	

* Contains naturally occurring sugars. † Weak spot. ‡ Small amount of lecithin.

The brown material isolated from emulsion containing soybean phosphatides appears to be the result of the reaction between dextrose and both lecithin and cephalin, as choline positive spots with R_f values of 0.52 and 0.80 were obtained on the chromatograms.

Staining of chromatograms of the brown material for unsaturated groups, long chain alkyl groups, and for organic phosphate esters resulted in spots at the same R_f values as those obtained for the ninhydrin positive and choline positive spots. The only exceptions were at those spots with R_f values <0.1. Therefore, the brown material did not contain just amino acids, amines or free choline as degradation products. Dextrose alone had an R_f value of 0.60.

Summary. This investigation gives experimental evidence that at autoclaving temperature (121°C) a type of browning occurs which involves reaction of dextrose with lecithins as well as with amino-groups of cephalins. Therefore, use of pure lecithin as an emulsifier in an oil emulsion containing dextrose in the water phase would not eliminate formation of the brown material. The complex browning reaction is responsible for formation of a colloidal material found in phosphatide-containing fat emulsions prepared for intravenous alimentation, and might be a

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Production of Mucopolysaccharides by Synovial Cells in a Simplified Tissue Culture Medium. (22853)

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Acid mucopolysaccharides of connective tissue ground substance and synovial fluid "mucin" have assumed increasing importance in the light of mounting evidence that these substances are abnormal in various rheumatic disease states(1,2). Information regarding specific cell or cells of origin of the several acid mucopolysaccharides is contradictory (3,4,5) and biochemical data concerning their biosynthesis in normal tissue are fragmentary. Since most of the information concerning biosynthesis has been obtained from study of microbiologic systems capable of synthesizing mucopolysaccharides(6), it would be desirable to initiate parallel exploration at the level of the mammalian tissue cell. While circumstantial evidence of production of mucopolysaccharide by synovial cells has been obtained by tissue culture methods, previous studies are open to the criticism that the mucopolysaccharides demonstrated may well have been present in the initial culture medium. As early as 1933, Vaubel(7) reported the production of synovial "mucin" by rabbit synovial tissue grown *in vitro* in tissue culture. The production of a mucin clot on acidification of the culture medium, which contained splenic extract, was taken as evidence of "mucin" production. Vaubel regarded this phenomenon as specific for the synovial cell. Murray *et al.*(8) subsequently successfully cultured adult rat synovium *in vitro*. Their observations tended to confirm Vaubel's belief that the synovial cell in tissue culture was morphologically distinct from the "common fibroblast." Although they did not study the products of cellular metabolism in their culture media, they were unable to confirm Vaubel's finding of metachromatic staining of the cytoplasm with toluidine blue. More recently Grossfeld *et al.*(9) advanced further evidence of *in vitro* mucopolysaccharide production using cultures of rat subcutaneous tissue as well as normal and arthritic human synovium. In the culture medium which had supported synovial cell growth they measured mucin clot formation and turbidity following acidification with acetic acid. They noted that these phenomena could be prevented by

* Fellow of National Arthritis and Rheumatism Foundation.

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prior treatment of the medium with either testicular or pneumococcal hyaluronidase. The culture medium used by these workers contained amniotic fluid, horse serum and, in most instances, chick embryo extract. Their observations suggested that hydrolysis of the embryo extract with hyaluronidase prior to its use as a medium component or omission of embryo extract from the nutrient medium adversely affected mucin clot production. Kling *et al.*(10) apparently were also troubled by the problem of incorporation of mucopolysaccharides into the initial culture medium and minimized this by reducing the embryo extract concentration to 2.5%. Their evidence for mucopolysaccharide production was documented by showing the presence in culture media of hyaluronidase-sensitive turbidigenic material, by mucin clot formation, by demonstrating a slight increase in viscosity of the culture medium and by showing an electrophoretic peak believed to be hyaluronic acid. It was of interest that they found no evidence of mucopolysaccharide production in cultures of para-articular tissues.

It was the purpose of the present study to detect mucopolysaccharide formation by human synovial cells using a simpler medium free of detectable preformed mucopolysaccharides and to examine the pattern of behavior of cultures from individuals of varying ages over a prolonged period of observation.

Materials and methods. Normal human synovial tissue was secured from knee joints included in amputations for peripheral vascular disease, osteogenic sarcoma, and from a 4½ month old fetus. The joints were normal by gross inspection and microscopic examination of the synovium. Less than 0.3 ml of synovial fluid was aspirated from the joints before tissue was removed for explantation. The excised tissue was rinsed with balanced salt solution(11) and cut into 1 x 1.5 mm explants. These tissue fragments were planted 6 to 10 per roller tube with a thin film of chicken plasma to improve adherence to the glass. The nutrient medium used contained 80% Eagle's basal medium(12), and 20% normal human serum. One-half ml of nutrient medium with an initial pH of approximately 7.8 was placed in each roller tube and the cultures were incubated at 37°C in a roller drum. Medium changes were carried out at 3-6 day intervals and the pH of culture media was adjusted with isotonic sodium bicarbonate when it approached pH 7.2. Frequent microscopic inspections of the roller tube cultures were made, and cover-slip cultures yielded preparations suitable for staining. The culture medium was analyzed for mucopolysaccharides by a turbidimetric method and also by a modified Sundblad‡ method for hexosamine. For turbidimetric analysis of each sample, 0.5 ml of culture medium was delivered to each of two 10 x 80 mm colorimeter tubes and adjusted to a pH of approximately 5.0 with 0.2 ml of 0.1 M acetate buffer, pH 4.91. 0.05 ml of 0.02 M phosphate buffer at pH 6.8 with 0.45% sodium chloride and 0.01% bovine serum albumin was added to the first tube and to the second tube was added 0.05 ml of the same buffer mixture containing 50 turbidity reducing units (TRU) of testicular hyaluronidase.§ Both tubes were incubated in a water bath at 37°C for 30 minutes and then turbidity was developed by addition of 0.3 ml of 1 M acetate buffer, pH 4.2. After standing for 30 minutes at room temperature, the optical transmission of the turbid samples was determined in the Coleman colorimeter, Model #9, with a 590 mu filter. The quantity of hyaluronidase-sensitive turbidigenic material in the culture medium was determined from a standard curve with purified umbilical cord hyaluronate as a reference (Fig. 1). The hyaluronic acid used as a reference standard

‡ Hexosamine was determined by extensive dialysis of the samples followed by treatment with hyaluronidase, precipitation of proteins with *hot* trichloroacetic acid, acid hydrolysis of the supernatant fluid, and analysis for hexosamine by the method of Boas (13) which involves purification by an ion exchange resin technic. The modifications of the original Sundblad method(14) indicated here were found to be necessary for accurate determination of hyaluronic acid in synovial fluid, and will be published elsewhere. The hexosamine analyses were performed through the courtesy of Mr. Donald Watson.

§ We are indebted to Wyeth Co. for the highly purified testicular hyaluronidase used.

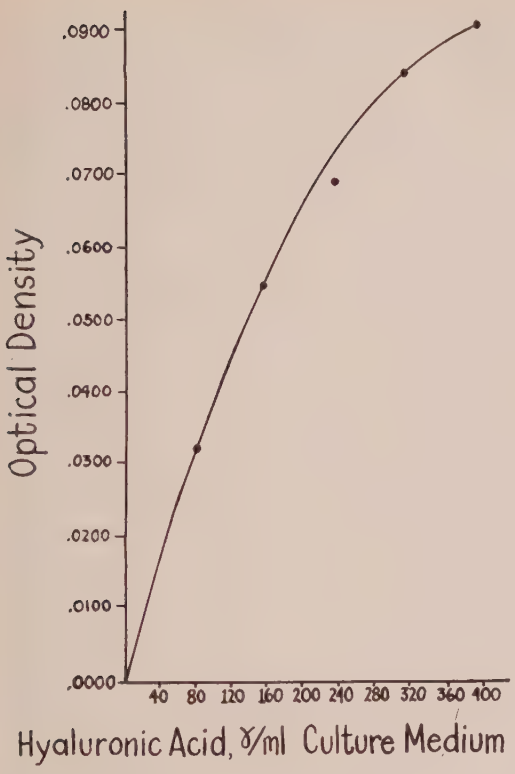

FIG. 1. Standard curve derived after adding reference hyaluronic acid to initial culture medium.

had been purified by electroconvection‖ and was quantitated on the basis of the hexosamine which it contained. The relative viscosity of the reference hyaluronic acid at a concentration of 2.8 mg/ml is approximately 32 at pH 7.0 in 0.03 M phosphate buffer.

Observations. Culture morphology and behavior. Group I cultures were derived from the suprapatellar bursa of a 67-year-old male patient with diabetes mellitus. For the first month these explants were cultured *in vitro* in a medium containing 10% embryo extract and for the succeeding 5 months they were grown in 80% Eagle's basal medium with 20% human serum. It was possible to subculture the cells mechanically five times, after which the cultures degenerated. The cellular morphology was pleomorphic, ranging from spindle and stellate shaped cells to more polygonal forms. In general, cytoplasmic granulation as demonstrated by the May-Greenwald-Giemsa stain was not marked, but a perinuclear halo of violet granules was commonly seen (Fig. 2). The pattern of growth was reticular and the rate of proliferation appeared slow. Lysis of the plasma clot was not uncommon.

Group II cultures were obtained from the knee synovium of a 14-year-old boy whose leg was amputated for osteogenic sarcoma, and were grown in the medium previously described. No subcultures were attempted. These explants showed a much more rapid initial rate of proliferation than those of Group I. This rapid outgrowth was not sustained and the cultures were lost after two months. Plasma clot lysis and decrease in pH of the medium were considerably more prominent in these cultures than in those from Group I. Cellular morphology did not differ appreciably from Group I.

Culture Group III was derived from the knee joint synovium of a 4½-month-old male fetus. The explants were removed from the area of insertion of the quadriceps complex into the patellar cartilage. Outgrowth around the fetal explants appeared at 72 hours and rapid proliferation of small granular connective tissue cells in a reticular pattern followed (Fig. 3 and 4). Concomitant with accelerated proliferation, the fetal culture produced

FIG. 2. Four-day cover slip culture of synovium from 67-yr-old male. May-Greenwald-Giemsa stain. × 170.

‖ Abstract by Roseman, Watson, Duff and Robinson in *Fed. Proc.*, 1955, 14, No. 1.

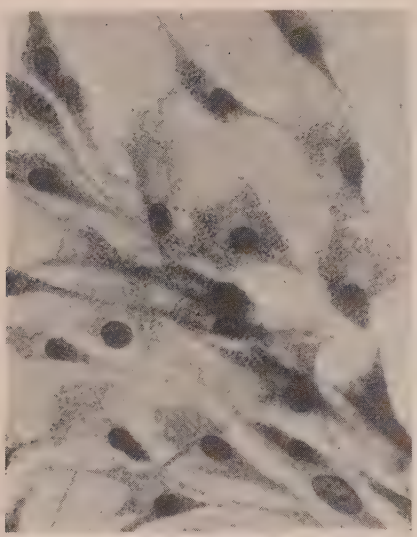

FIG. 3. Three-day cover slip culture of synovium from 4½-mo-old fetus. May-Greenwald-Giemsa stain. × 170.

more acid than previous strains. Mechanical division of the culture for one subculture was successful. Culture degeneration and death occurred after 6 weeks.

Pattern of mucopolysaccharide synthesis. The quantity of hyaluronidase-sensitive polysaccharide found in the culture media of 3 groups of human synovial explants is tabulated (Table I). From the table it is apparent that in our hands the turbidimetric method shows no mucopolysaccharide in the simplified culture medium *per se* in contrast to large amounts found when embryo extract is a component of the medium. Turbidimetric measurement of polysaccharide synthesis by explants grown in a medium containing embryo extract would appear to be open to serious question, since small increases measured by difference would be of doubtful sig-

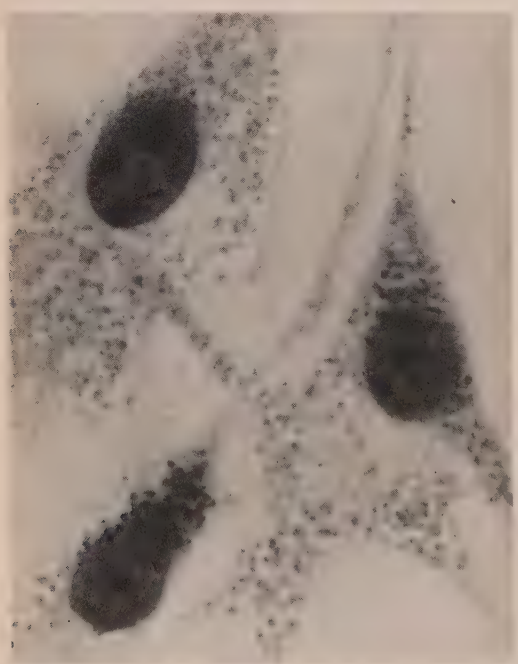

FIG. 4. Same culture as Fig. 3. × 756.

TABLE I. Hyaluronic Acid Content of Culture Media.

Culture group	Medium	% EE50*	Incubation time of medium tested, days	Time post explantation, days	Hyaluronic acid, γ/ml†
None	$ESM_{80}HS_{20}$	20	0	0	1540
	$AF_{80}HS_{20}$‡	20	0	0	1080
	$ESM_{80}HS_{20}$§	0	0	0	0
I	$858_{50}HS_{50}$‖	0	6	49	80
	$ESM_{80}HS_{20}$	0	4	97	36
		0	4	101	76
		0	4	172	84
		0	3	175	72
		0	4	179	72
		0	4	183	60
		0	4	187	60
II	$ESM_{80}HS_{20}$	0	9	15	244
	$ESM_{60}HS_{40}$	0	7	32	95
	$ESM_{80}HS_{20}$	0	5	37	132
		0	4	41	112
		0	4	45	128
		0	3	48	120
		0	4	52	104
		0	4	56	108
		0	4	60	172
III	$ESM_{80}HS_{20}$	0	8	8	519
		0	7	15	264
		0	5	20	168
		0	4	24	120
		0	4	28	76
		0	3	31	136
		0	4	35	40
		0	4	39	38
		0	4	43	28
		0	3	46	84

* % EE50 indicates % of medium represented by half diluted chick embryo extract (Difco).

† Hyaluronic acid determined by turbidimetric technic described.

‡ $AF_{80}HS_{20}$ represents amniotic fluid, 60%, human serum, 20%.

§ $ESM_{80}HS_{20}$ refers to 80% Eagle's basal medium with 20% human serum.

‖ $858_{50}HS_{50}$ indicates Parker's medium 858(16) diluted 1:1 with human serum.

nificance. In all 3 culture groups mucopolysaccharide appeared to be released by the cell cultures into the nutrient medium. For each culture group the first few days *in vitro* showed the greatest polysaccharide yield with a tendency to a lower, relatively constant level of polysaccharide production in later weeks.

Criteria for identification of turbidigenic substance as mucopolysaccharide. Culture media which have supported the *in vitro* maintenance and proliferation of synovial connective tissue cells contain one or more substances which develop turbidity on acidification. Treatment of the sample with testicular hyaluronidase will prevent or markedly reduce such turbidity formation. Positive identification of the turbidigenic substance as a mucopolysaccharide would follow only if it were possible to use pure hyaluronidase. Testicular hyaluronidase with 1250 TRU/mg obviously may contain other enzymes capable of hydrolyzing turbidigenic materials. We have noted that 50 TRU of testicular hyaluronidase is very active in preventing turbidity formation by several hyaluronate and chondroitin sulfate preparations. The testicular hyaluronidase proved to be contaminated with a significant amount of ribonuclease since it was shown to be active against ribonucleic acid. No activity against desoxyribonucleic acid could be demonstrated. The testicular hyaluronidase used in this study has equivalent activity against added hyaluronic acid in both initial culture medium and that which had supported cell growth. Fifty gamma of crystalline ribonuclease was shown to have equivalent turbidity-preventing activity against ribonucleic acid added to both initial medium and medium which had been incubated with metabolizing synovial cells. Crystalline ribonuclease had, however, no capacity to inhibit the turbidity formation found in culture media which had nourished synovial cells. These observations indicate that the test culture media do not contain ribonucleic acid in a significant quantity and suggest that the turbidigenic material is a mucopolysaccharide. To further substantiate the belief that the turbidigenic material in synovial culture media is a mucopolysaccharide, selected samples were analyzed for hexosamine. Table II compares the quantity of hyaluronic acid in samples of media as measured by the turbidimetric and hexosamine methods.

TABLE II. Comparison of Hyaluronic Acid Values Measured by Hexosamine and Turbidimetric Methods.

Culture group	γ hyaluronic acid/ml by hexosamine method	γ hyaluronic acid/ml by turbidimetric method	% of turbidimetric value accounted for by hexosamine
$ESM_{80}HS_{20}$	.0	.0	
I	8.7	72	12
II	28.9	108	27
	28.9	132	22
III	48.1	264	18
	25.5	136	18

The turbidimetric and chemical data support the thesis that synovial cells will release a mucopolysaccharide into the culture medium, but do not allow one to distinguish between hyaluronic acid and chondroitin sulfate as the substance produced. To aid in this distinction we prepared bacterial hyaluronidase from the filtrate of cultures of *Clostridium perfringens*¶ using the technic described by Baker *et al.*(15). This bacterial hyaluronidase completely prevented turbidity formation of synovial fluid hyaluronate added to initial culture medium but was not detectably active against chondroitin sulfate added to initial culture medium. *Clostridium perfringens* hyaluronidase proved capable of preventing turbidity formation in the dialyzed culture medium which had supported synovial cell growth, thus supporting the likelihood that the mucopolysaccharide present is hyaluronic acid.

Discussion. From the turbidimetric and chemical data it appears that *in vitro* cultures of normal human synovial tissue can produce acid mucopolysaccharide from the constituents of a semi-synthetic medium. The medium found satisfactory here was Eagle's basal medium (containing 12 amino acids,

¶ We are indebted to Dr. Donald J. Merchant of our Medical School for the strain of *Clostridium perfringens* used.

vitamins, l-glutamine, electrolytes and glucose) supplemented with 20% human serum. Although isolation and final chemical characterization of the mucopolysaccharide is not practicable with the small quantity of medium available here, the data are consistent with the belief that hyaluronic acid is the substance found in the culture media. The substantially greater quantities of mucopolysaccharide found by turbidimetry than by the Sundblad hexosamine method might be explained in at least three ways: (1) the hyaluronate used as a reference standard may have been sufficiently denatured in the process of isolation so as to yield less turbidity per unit of hexosamine than the material produced by the synovial cell culture; (2) a substantial proportion of the turbidigenic material may not be hexosamine-containing mucopolysaccharide; (3) the dialyzable mucopolysaccharide fraction of the culture medium, which is not measured by the Sundblad hexosamine method, may actually contribute to the higher turbidimetric values.

It must be remembered that the "synovial" cultures described here and elsewhere are imperfectly characterized both qualitatively and quantitatively. Hence, the possible mixture of cell types within impure strains, as well as unmeasured variations in number of cells acting on a substrate, subjects the critical comparison of different cell strains to many uncertainties. It is apparent that the similarities in mucopolysaccharide concentration among the three groups of cultures related to time post-explantation may just as easily be an artifact arising from differences in number of metabolizing cells as evidence of similar biosynthetic capacities of synovial cells from individuals of different ages.

Summary. Synovial tissue has been shown to be capable of *in vitro* production of a mucopolysaccharide believed to be hyaluronic acid. Cultures from donors ranging from a fetus to an elderly male shared this capacity for periods of 6 weeks to 6 months. This process is supported by a nutrient medium that is 80% defined and excludes embryo extract. Culture behavior and cellular morphology are described.

The author wishes to express his appreciation to Dr. Donald J. Merchant, Dept. of Bacteriology and to Dr. William D. Robinson and Dr. Ivan F. Duff, Dept. of Internal Medicine, Univ. of Michigan Medical School, for their helpful advice. Dr. Saul Roseman, Dr. Eugene A. Davidson and Dr. Harold J. Blumenthal were helpful in solving the many chemical problems. We are grateful to the operating room nursing staff and the Dept. of Pathology, who aided in securing viable specimens.

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A Renal Action of Glucagon (22854)

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In the course of a study on the fate of I^{131}-glucagon *in vivo* it was observed that a considerable portion of the protein bound I^{131} appeared rapidly in the urine as free iodide.* Furthermore, this excretion of inorganic I^{131} was 3 or 4 times greater than when an equivalent amount of inorganic I^{131} alone was injected. This suggested that glucagon had accelerated the excretion of the liberated iodide. For this reason a study was undertaken to determine the effect of glucagon not only on the renal excretion of I^{131}, but on a number of other electrolytes.

Procedure and methods. Adult mongrel dogs of both sexes were used in the experiments to be described. Unless otherwise specified, the animals were anesthetized with sodium pentobarbital (Nembutal) and diuresis induced by a constant infusion of 5% glucose solution at a rate of 3 to 5 ml/min. These conditions yielded a fairly constant urine flow of at least 2 ml/min after 2 to 3 hours of infusion. Electrolyte excretion was determined by analysis of urine samples collected at specific time intervals through an indwelling bladder catheter. The bladder was washed once with 10 ml of distilled water and the wash added to the urine sample. During the control period the urine samples were collected at 30 min intervals for 90 minutes followed by four 15 minute collections. Glucagon was then administered intravenously and the urine collected again at 15 minute intervals for 1 hour followed by three 30 minute samples. The total duration of the experiment was 5 hours. Chloride, inorganic phosphate, urinary reducing substance, and creatinine clearances were determined by standard methods(1). Sodium and potassium were analyzed on an internally compensated flame photometer.

Results. Table I summarizes the excretion data from the 19 experiments. The variability of the magnitude of the glucagon effect is obviously very great. However, the overall average increases in excretion of all electrolytes studied are very marked, ranging from 207 to 510% over control values. No consistent correlation between the magnitude of the effect and the dose of glucagon in this relatively small number of experiments is apparent.

The effect of glucagon on renal excretion of electrolytes was studied in 7 dogs using a single intravenous dose ranging from 30-300 γ/kg body weight of a 50% pure preparation.† Fig. 1 and 2 represent a typical experiment showing the effect on Cl^-,PO_4^{---}, Na^+ and K^+ excretion. The upper curves illustrate the cumulative output of electrolytes as a function of time. It is evident from these graphs that per unit time output of all electrolytes increases several fold shortly after administration of glucagon. As shown by the lower curve of Fig. 1 the urine flow expressed as ml/min remained fairly constant throughout the experiment. Although the experiments were designed to keep the urine flow as constant as possible, some fluctuation was unavoidable. It is unlikely, however, that these limited variations are responsible for changes in electrolyte output since it has been shown by Wesson and coworkers(2) that the excretion of Na^+ and Cl^- is independent of urine flow in the range from 1 to 6 ml/min. Furthermore, an increase in ion excretion was frequently observed while urine flow was decreasing. The pH of all urine samples was measured in several experiments; no signifi-

* Unpublished observations.

† A 50% pure glucagon preparation (Lot No. 208-158B-197) was kindly supplied by Dr. O. K. Behrens of the Lilly Research Laboratories, Indianapolis. The crystalline glucagon was prepared from such material according to the method of Staub *et al.* (*J. Biol. Chem.*, 1955, v214, 619.)

TABLE I. Effect of Glucagon on Renal Excretion of Electrolytes in the Dog. Summary of electrolyte excretion data showing avg changes and variability of results comparing control periods with experimental (glucagon) periods.

Glucagon,* γ/kg body wt	No. of dogs	Na^+ Avg increase, %	Na^+ Range, %	K^+ Avg increase, %	K^+ Range, %	Cl^- Avg increase, %	Cl^- Range, %	PO_4 Avg increase, %	PO_4 Range, %	I^{131} Avg increase, %	I^{131} Range, %
30–100	6	249	0 → 758	184	0 → 489	42	0 → 130	152	0 → 843	0	0
100–200	6 or 7†	581	85 → 855	243	43 → 676	459	0 → 1670	598	61 → 2163	205	0 → 754
200–300	5 or 6†	1069	0 → 4152	192	76 → 481	810	0 → 1450	529	16 → 1158	531	27 → 1241
Overall avg increase, %	17 or 19		510		207		409		399		252

* Dose expressed as potency of the crystalline glucagon preparation.
† Electrolyte measurements incomplete in one animal.

cant change was observed after the administration of glucagon.

Endogenous and in some instances exogenous creatinine clearances were determined in all experiments as a measure of the glomerular filtration rate. Glucagon had no significant effect on the glomerular filtration rate, the variations being in most cases within ± 10%. This suggests that the polypeptide acts at the renal tubular level rather than at the glomerulus.

The same effects were observed in 12 dogs in which crystalline glucagon was used instead of the 50% pure preparation. No effects were observed in one animal which received a heat inactivated preparation of glucagon and in another animal to which insulin was administered. For these reasons it is unlikely that the described effects can be attributed to an impurity in the glucagon preparation or to a non-specific protein effect.

To rule out anesthesia as a factor influencing renal function after glucagon administration, several experiments under varying conditions were repeated in a trained, unanesthetized female dog. Fig. 3 summarizes the effect of glucagon on the excretion of Na^+ under these conditions. All other electrolytes tested behaved in a similar manner. In the experiment illustrated by curves "A", 5% glucose was infused as usual and the urine flow was approximately 2 ml/min. Another experiment was performed on a different day with no water load as is evident by the very low urine flow illustrated by the lower "B" curve. The results provide evidence that neither anesthesia nor moderate diuresis is a necessary condition for the effect of glucagon on electrolyte excretion.

It is known that large amounts of glucagon cause prolonged hyperglycemia with occasional glycosuria(3). It is conceivable, therefore, that the high blood sugar levels and/or glycosuria might affect the pattern of electrolyte excretion. To test this possibility, plasma glucose levels of 300-500 mg % were established by a priming dose of glucose (1 g/kg) and infusion of 12.5-15% glucose solution. These conditions caused consistent glycosuria as shown by the lower curve of Fig. 4.

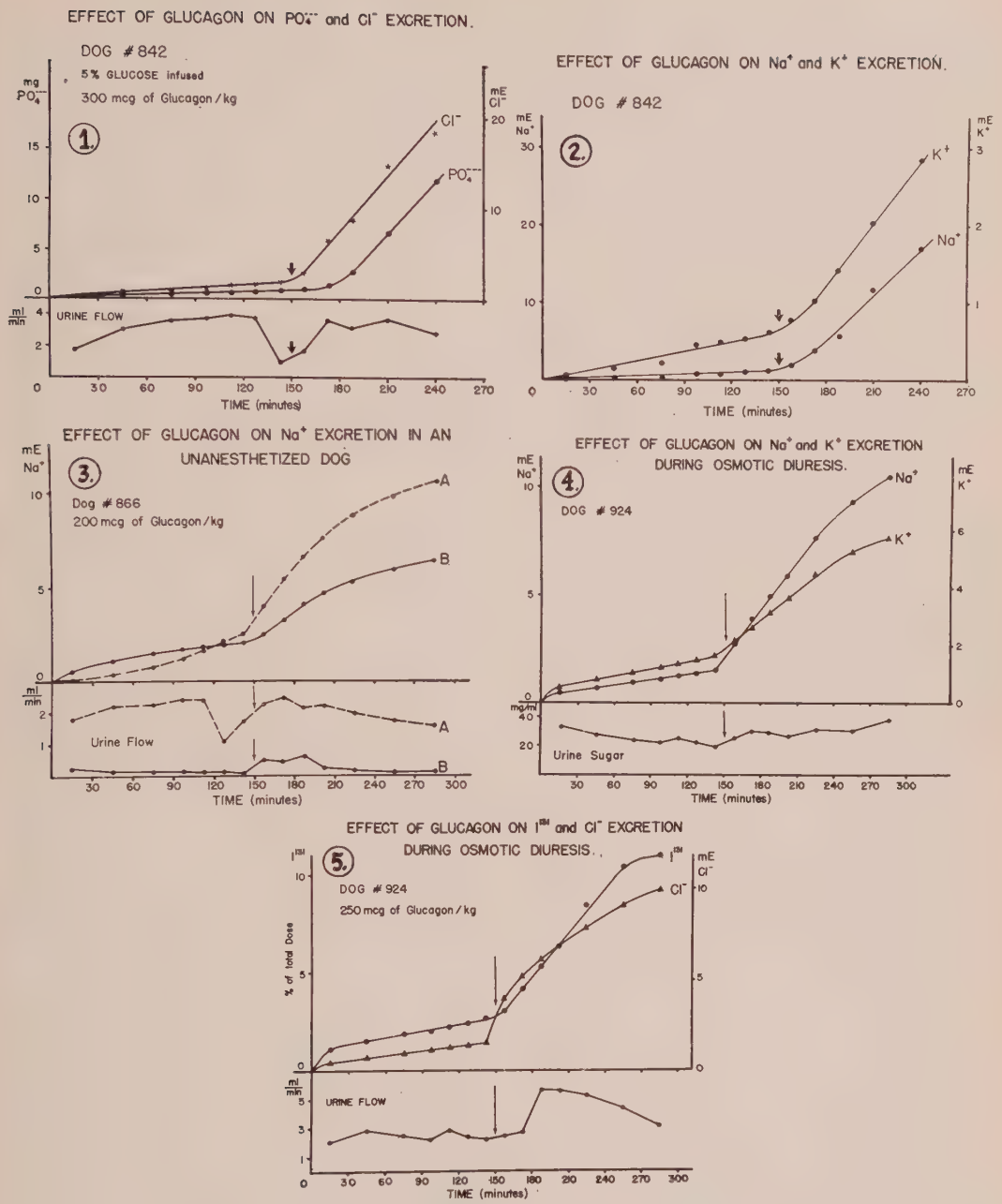

FIG. 1-5.

It was found (Fig. 4 and 5) that even in animals with pre-existing hyperglycemia and glycosuria, glucagon has a marked enhancing effect on the urinary excretion of I^{131}, Cl^-, Na^+ and K^+. Consequently it appears that hyperglycemia and glycosuria *per se* are not responsible for the described electrolyte changes.

Summary and conclusions. It has been shown that glucagon has a marked enhancing effect on renal excretion of I^{131}, Na^+, K^+, Cl^- and PO_4^{---} in both the anesthetized and con-

scious dog. This action of glucagon, which is independent of its hyperglycemic effect, appears to be due to a direct action on the kidney tubules. The physiologic significance of these effects remains to be clarified.

We wish to thank Dr. C. G. Mackenzie and Dr. J. H. Holmes for their interest and advice.

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Some Factors Which Influence Vesiculase Action. (22855)

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A previous publication(1) described coagulation of proteins of the seminal vesicle secretion by vesiculase, an enzyme of prostatic origin. In experiments conducted at room temperature, coagulation was prevented by ethylenediamine tetraacetic acid (versene), and certain other metal chelating agents. Versene inhibition could be reversed by addition of Mn^{++} ions, and somewhat higher concentrations of Ca^{++} ions, but not by Mg^{++} ions. Heavy metal ions, *e.g.*, Hg^{++}, also abolished coagulation. This paper describes experiments which give further insight into the role of metal ions in the coagulation of semen. The influence of temperature and of a number of substances on the over-all coagulation process has also been studied.

Methods. Turbidity measurements were made with a Coleman Junior spectrophotometer using cuvettes of 0.8 cm light path. Other spectrophotometric measurements were made with Beckmann model D.U. spectrophotometer using quartz cells of 1 cm light path. Protein was determined spectrophotometrically according to Warburg and Christian(2) or by the method of Lowry *et al.*(3). Nitrogen was estimated by the Kjeldahl technic. Dry weights were determined by heating to constant weight at 105°. Total phosphorus was estimated after digestion with sulfuric acid, subsequent clarification with H_2O_2 and hydrolysis in 1 N acid for 10 minutes at 100°, by determination of inorganic phosphorus according to Gomori(4).

Materials. Crystalline proteolytic enzymes, takadiastase and hyaluronidase were of commercial origin. Five times recrystallized soy bean trypsin inhibitor was obtained from Nutritional Biochemicals Corp. 2,3 dimercaptopropanol was purchased from Mann Research Laboratories. Spermine hydrochloride was generously donated by Dr. E. A. Evans, Jr. Heparin (60 units/mg) was obtained from Parke, Davis and Co. Acetone-desiccated proteins of seminal vesicle secretion were prepared as described elsewhere(1). For coagulation studies, the acetone powders were extracted with isotonic NaCl, and a small insoluble residue removed. Ultraviolet absorption spectra of these saline extracts were identical in either phosphate or tris (hydroxymethyl) aminomethane (Tris) buffers, and also in 6.4 M urea, at pH 7. The ratio of absorbancy at 280 mμ to that at 260 mμ ranged from 1.24 to 1.53 (mean 1.38). Mean absorbancy at 280 mμ was 2.64/mg nitrogen/ml and 0.43/mg dry weight/ml. Phosphorus content of acetone dried preparations varied from 0.02% to 0.03%. Crude preparations were obtained by homogenizing fresh coagulating glands in isotonic NaCl, followed by centrifugation at 5,000 x g for 20 minutes at 2°. Partially purified preparations were obtained by fractionation of crude extracts

* Scholar in Cancer Research of Am. Cancer Soc.

with ammonium sulfate between 0.3 and 0.5 saturation, followed by fractionation with acetone between 0% and 30% (5). All fractionations were carried out at less than 2°, and the fractions were dissolved in and dialyzed against isotonic NaCl. Ultraviolet absorption spectra of these partially purified preparations showed a distinct peak at 280 mμ; the ratio of absorbancy at 280 mμ to that at 260 mμ was in the neighborhood of 1.75. Both crude and partially purified preparations of guinea pig vesiculase retained their activity for many weeks when stored frozen, and for several days when stored at 2°. Repeated freezing and thawing, however, was deleterious to the enzyme. *Optical Determination of Coagulation.* The coagula formed by action of vesiculase formed a firm gel only when initial concentration of seminal vesicle proteins was very high. For this reason it was not feasible to determine the end point of coagulation by inversion of the tube. Rate and extent of coagulation was followed by measurement of change in turbidity at 660 mμ. The reactions were carried out in a final volume of 1.0 to 1.5 ml. Unless stated otherwise, the reaction was initiated by addition of vesiculase in 0.1 ml or less. After all components were added, the end of each tube was covered with a sheet of "parafilm" and contents mixed by slow inversion 3 times. Optical density readings were taken after mixing, and at suitable time intervals thereafter. In all experiments blank tubes were set up in which either seminal vesicle proteins or vesiculase were omitted, to correct for any turbidity changes in absence of these components. Vesiculase preparations by themselves never resulted in turbidity formation. The experiments were carried out at pH 7.0-7.4 in either Tris or phosphate buffers. Final ionic strength was adjusted to approximately 0.12 with NaCl, and the final concentration of seminal vesicle proteins was approximately 20 mg/ml. The term "standard conditions" describes reaction mixtures of this type. The final optical density in such systems was in the vicinity of 1, and the lag period was defined arbitrarily as time (in minutes) required to reach an optical density of 0.1. Under these conditions changes in turbidity reflected the formation of a uniform, opaque coagulum. Turbidity changes in duplicate tubes invariably agreed within 5% of one another. Change in light absorption at 400 mμ was 1.5 to 1.8 times greater than that observed at 660 mμ, but followed the same rate of change with time. All experiments were performed with vesiculase and seminal vesicle proteins prepared from guinea pigs weighing between 450 and 600 g.

Results. Temperature and coagulation. In experiments of less than 4 hours duration, no insoluble material was formed from seminal vesicle proteins without addition of vesiculase below 30°. However, at 38°, a slow formation of insoluble material occurred in the absence of this coagulating enzyme under standard conditions. This "spontaneous" coagulation of seminal vesicle proteins occurred more rapidly, and to a greater extent, if the ionic strength was less than 0.10. However, final turbidity, observed without addition of vesiculase was, under standard conditions,

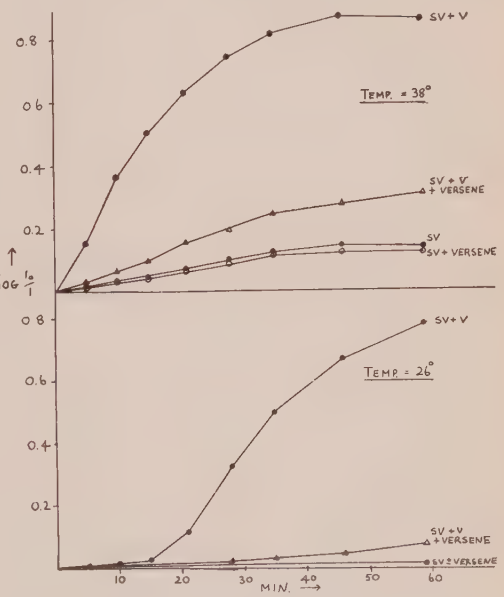

FIG. 1. Influence of temperature on vesiculase activity. Each vessel contained 50 μM Tris buffer of pH 7.4; 90 μM NaCl; 25 mg seminal vesicle protein and 0.7 mg vesiculase protein in a final vol of 1.2 ml. SV = seminal vesicle protein added; V = vesiculase protein added. Disodium versenate, if added: 0.3 μM. No change in optical density occurred at either temperature when vesiculase was added in the absence of seminal vesicle protein.

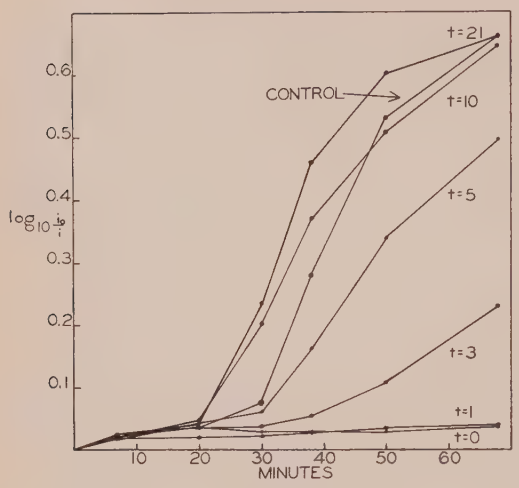

FIG. 2. Delayed addition of versene. Each cuvette contained 50 μM Tris buffer of pH 7.45; 105 μM NaCl; 25 mg seminal vesicle protein and 0.17 mg semi-purified vesiculase, in total vol of 1.42 ml. Disodium versenate (0.5 μM) added in vol of 0.02 ml. t = time in min. elapsing between mixing of all components and addition of versene. Temp. 25°.

usually less than one-tenth of that observed in the presence of vesiculase. Fig. 1 shows that the "spontaneous" coagulation at 38° was quite insensitive to versene, whereas, the vesiculase-induced coagulation was sensitive to versene at either 26° or 38°. Raising the temperature between these limits decreased the lag period, but had much less influence upon the rate of turbidity change once coagulation was initiated.

Inhibition by versene and by Hg^{++} ions. Previous experiments(1) demonstrated that the inhibitory action of versene on coagulation could be counteracted by Mn^{++} or Ca^{++} ions, neither of which affected vesiculase action in the absence of this chelating agent. Further insight into the role of divalent metal ions in the coagulation process was afforded by experiments in which vesiculase was allowed to react with seminal vesicle proteins for short periods of time, after which inhibitors were added to the reaction mixture. These experiments were conducted at ionic strengths such that no coagulation occurred during the period of preincubation of vesiculase (*cf.* 1). Typical experiments illustrated in Fig. 2 show that preincubation with vesiculase in the absence of versene for one minute did not permit coagulation to occur. However, preincubation with the enzyme for as little as 3 minutes resulted in coagulation even after addition of versene in concentrations sufficient to abolish coagulation when added prior to vesiculase. Fig. 3 shows that Hg^{++} ions, which also inhibited the over-all coagulation process, act in a manner different from versene. Thus, even after 10 minutes preincubation of seminal vesicle proteins with vesiculase, no coagulation took place following addition of Hg^{++} ions. Clearly, two distinct reactions are involved in the coagulation process. The first of these is sensitive to versene and does not necessarily involve preincubation of insoluble material, whereas the second, versene-insensitive stage leads to formation of a coagulum and is inhibited by Hg^{++} ions.

Miscellaneous agents. The following substances were without influence upon vesiculase-induced coagulation determined under standard conditions at 28°: spermine hydrochloride (0.001 M); glucose (0.04 M); glycerol (0.08 M) (*cf.* 6); 2,4-dinitrophenol (0.0007 M); crystalline soy bean trypsin inhibitor (0.07 mg/ml) and heparin (0.6 unit/ml). Higher concentrations of heparin caused precipitation of seminal vesicle proteins in the absence of vesiculase. Although cysteine (0.002 M) was without influence

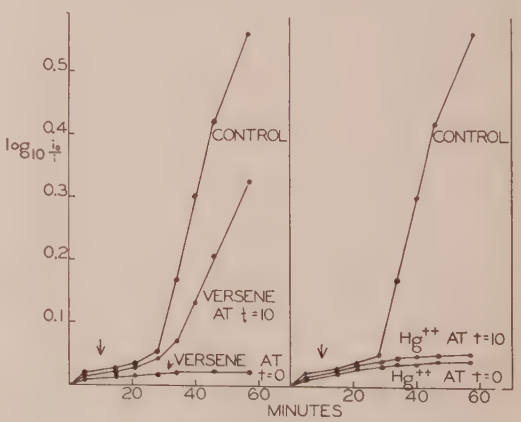

FIG. 3. Delayed addition of versene and of Hg^{++} ions. Each vessel contained 50 μM Tris buffer of pH 7.4; 25 mg seminal vesicle protein; 120 μM NaCl and 0.17 mg semi-purified vesiculase, in total vol of 1.42 ml. Disodium versenate (0.5 μM) or $HgCl_2$ (0.6 μM) added in vol of 0.02 ml. t = time in min. elapsing between mixing of all components and addition of inhibitors. Temp. 26°.

upon coagulation, and reversed the inhibitory action of Hg^{++} ions (0.00003 M)(1), low concentrations of 2,3-dimercaptopropanol (0.0002 M) abolished coagulation. At a final concentration of 0.0006 M, coagulation was unaffected by sodium citrate, adenosine triphosphate and sodium pyrophosphate. Higher concentrations of the latter substances inhibited coagulation, but it was not possible to ascertain whether this inhibitory action was due to their metal chelating properties, or to an increase in ionic strength resulting from addition of these multivalent ions. Profound inhibitory effects of high ionic strengths on vesiculase-induced coagulation of seminal vesicle proteins have been recorded elsewhere(1).

Attempts to induce coagulation with proteolytic and other enzymes. Many attempts were made to induce coagulation of seminal vesicle proteins in Tris buffer of pH 7.4 at high and at low ionic strengths by addition of crystalline or highly purified proteolytic enzymes. Over a concentration range of 0.005 to 1 mg/ml, the following enzymes failed to induce coagulation over a period of 4 hours at 25°: pepsin, trypsin, chymotrypsin, carboxypeptidase and papain. Moreover, addition of either hyaluronidase or takadiastase failed to induce coagulation. It has been reported previously(1) that thrombin will not clot seminal vesicle proteins. The coagulated material formed by action of vesiculase upon seminal vesicle proteins, after many washes with isotonic NaCl, was readily digested by trypsin and by chymotrypsin, and more rapidly by a mixture of these 2 proteolytic enzymes. Ultraviolet absorption spectra of these proteolytic digests (corrected for small absorption of the added proteolytic enzymes) showed distinct peaks at 280 mμ, and were fully consonant with the original protein nature of the coagula. Hyaluronidase was devoid of action upon the insoluble material produced by vesiculase action.

Discussion. The above experiments agree with previous reports(1,5) that coagulation of seminal vesicle proteins by vesiculase is an enzymatic denaturation of protein which bears little resemblance to the clotting of fibrinogen by thrombin. It is well established that in the latter reaction, the type of clot formed is dependent, *inter alia*, upon the composition of the medium employed. The ionic strength, pH and temperature affect in a most striking manner the opacity, tensile strength and electron microscopic structure of fibrin clots(6,7). Thus, it must be emphasized that under different experimental conditions, the turbidity changes resulting from vesiculase action upon seminal vesicle proteins may well reflect differences in the physicochemical state of the denatured proteins formed rather than differences in the primary action of vesiculase itself.

There is good evidence that the primary action of thrombin upon fibrinogen is proteolytic in nature. The ability of thrombin to hydrolyze tosyl-L-arginine methyl ester (TAMe) parallels its clotting activity, and TAMe itself inhibits coagulation of fibrinogen by thrombin(8). Vesiculase is not inhibited by either TAMe(1) or by soy bean trypsin inhibitor, which suggests that its primary action is different from thrombin or fibrinogen. Other studies(5) have emphasized that prostatic enzyme(s) capable of hydrolyzing TAMe are not identical with vesiculase. There is evidence that the action of thrombin upon fibrinogen leads to formation of acid-soluble peptides(9). Attempts to demonstrate liberation of acid-soluble nitrogen containing or ninhydrin reacting substances during coagulation of seminal vesicle proteins by vesiculase have been unsuccessful. However, the methods employed may not have been sufficiently sensitive to detect such materials. It is of interest that all of the proteolytic enzymes tested failed to induce clotting of the seminal vesicle proteins.

The first stage of vesiculase action appears to require a metal ion which is effectively chelated by versene, α, α'-dipyridyl and o-phenanthroline, and which can be replaced by Mn^{++} or Ca^{++} ions, but not by Mg^{++} ions. The nature of the "natural" activating metal cannot be ascertained from these studies. The substance bound by the metal chelating agents which inhibit coagulation appears to be present in the preparations of

seminal vesicle proteins rather than in the vesiculase preparations used, since coagulation induced by partially purified vesiculase, which had undergone repeated precipitation and dialysis, is not influenced by the addition of Mn^{++} or Ca^{++} ions to the reaction mixture (1).

Summary. Studies are presented on the influence of temperature and of a number of chemical substances upon coagulation of proteins of the seminal vesicle secretion by the prostatic enzyme vesiculase. Experiments involving delayed addition of metal chelating agents and of heavy metal ions have shown that the coagulation process can be separated into two distinct stages.

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Comparative Metabolism of Sr^{89} and Ca^{45} by Bone Grown *in vitro*.* (22856)

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The comparative metabolism of calcium and strontium has been of recent interest because of the possible hazards arising from ingestion of radioactive strontium. Calcium has been shown to be preferentially absorbed from the digestive tract(1) and strontium to be excreted to a greater extent than calcium by the kidney(1,2); the net result of both processes favors the retention of calcium over strontium. Evidence is lacking, however, that bone metabolism distinguishes between these two alkaline earth metals. The large discriminations of the intestinal tract and the kidney could mask any selective capacity of bone when the intact animal is used as the experimental subject. Therefore, embryonic chick bones were cultured *in vitro* in order to study the comparative metabolism of Sr^{89} and Ca^{45} by bone uninfluenced by the action of the other organs.

Most experiments have compared calcium to carrier-free radiostrontium. It seemed advisable for present purposes to include the uptake of radiostrontium in the presence of significant levels of carrier strontium. This would also have clinical aspects since stable strontium salts have been advocated as therapeutic agents to aid in the remineralization of bone(3).

Methods. The procedure used was essentially that of Fell(4) except that the bones, rather than resting in a plasma clot, were bathed by a liquid medium while in a roller-tube apparatus. Tibias and femurs were dissected from 7-day-old chick embryos and adhered to the walls of 18 x 150 mm pyrex test tubes in a chicken plasma clot. The medium consisted of 0.5 ml chick embryo extract, 2. ml of dog serum, 50 μl of Tyrode's solution containing about 0.1 and 0.05 μc of Ca^{45} and Sr^{89}, respectively, 100 units of penicillin, and 100 μg of streptomycin. The total amount of medium was increased by 50 μl in the experiments testing the effects of carrier strontium. The additions were made to the medium from

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0, 0.00037, 0.0037, and 0.037 M solutions of $SrCl_2$. The calcium content of the media ranged from 9.7 to 12.7 mg %. The prepared tubes were incubated at 37°C while being rotated at 20 revolutions per hour. The medium was changed every 2 or 3 days. Each bone was individually assayed for radioactivity. To prepare the samples, the bone was placed in a 1-inch diameter stainless steel planchet, dissolved in 1 ml of 6 N nitric acid, and dried under infra-red lamps. Self-absorption corrections were unnecessary since there was less than 1 mg of residue. The samples were counted, after a 21-day waiting period, under a thin window G. M. tube by means of a differential absorption of the β emissions(5). The results are expressed as the strontium-calcium Observed Ratio ($OR_{bone\text{-}medium}$) as suggested by Comar *et al.*

$$(1).\ OR_{bone\text{-}medium} = \frac{Sr^*/Ca^* \text{ of bone}}{Sr^*/Ca^* \text{ of medium}} (1).$$

Results. Experiment A presents the data on the Observed $Ratio_{bone\text{-}medium}$ (O.R.) of embryonic chick bone after immersion in the labeled medium for a 2-hour period. No distinctions were made between tibias and femurs since their relative metabolism of strontium and calcium were identical. In this early phase of mineral uptake, 1.08 times as much strontium-89 entered the bone as calcium-45. It will be noted that the carrier strontium at the 0.1 mg % level had no effect upon the total amount of Ca^{45} accumulated. About 0.16% of the Ca^{45} present in the medium was deposited in the bones under these conditions.

When bone was grown in the presence of the labeling isotopes for a period of 14 days an O.R. of 0.83 resulted (Exp. B). In preliminary experiments, culture periods as short as 7 days were sufficient to produce this ratio, indicating that equilibrium had been established between bone and the medium. When bone was grown in the labeled medium for 9 days and followed by 5 additional days in a radioisotope-free medium, the O.R. fell from 0.83 to 0.76. These ratios were shown to be different ($P<0.05$) by statistical analysis.

In another experiment employing a labeling period of 6 days, the O.R. was found to be 0.80, 0.71, and 0.66 at 0, 4, and 6 days, respectively, after being placed on the non-labeled medium. A plot of the Observed Ratios versus Time suggested that during this short period of observation the rate of decline was linear.

TABLE I. Deposition of Ca^{45} and the Strontium-Calcium Observed Ratio of Embryonic Chick Bone Grown *In Vitro*.

Carrier strontium added, mg %	Time period	No. of bones	Observed Ratio	Avg % of Ca^{45} dose in bone
		Exp. A		
.0	2 hr	24*	1.08 ± .02†	.16
.1	2	24	1.05 ± .01	.15
		Exp. B		
.0	14 days	30	.83 ± .02	4.95
.0	9	18	.84 ± .01	4.78
.0	14 ‡	30	.76 ± .01	3.00
		Exp. C		
.0	9 days	18	.84 ± .01	4.78
.001	9	18	.80 ± .02	4.38
.01	9	18	.80 ± .02	3.48
.1	9	18	.77 ± .02	2.23

* Composed of equal numbers of femurs and tibias assayed individually.
† Mean ± stand. error.
‡ Radioactivity omitted from the medium during the last 5 days.

In 9 days the bones (Exp. B) accumulated an average of 4.78 and 3.98%, respectively, of the radiocalcium and radiostrontium present in the medium. After 5 subsequent days without activity, only 3.00% of the Ca^{45} and 2.27% of the Sr^{89} remained. The Sr^{89}/Ca^{45} ratio of the lost activity was 0.96. If it is assumed that the ratio in bone declined linearly during this period, then by dividing 0.96 by the average strontium-calcium ratio of bone $\left(\frac{0.83 + 0.76}{2}\right)$ it can be calculated that 1.2 times as much strontium-89 left bone as calcium-45.

The data of Exp. C demonstrated that even with relatively high levels of non-radioactive strontium in the medium, bone retained more calcium-45 than strontium-89. Only at the 0.1 mg% level of carrier strontium was the O.R. depressed sufficiently to be significantly

different ($P<0.05$) than the O.R. of the carrier-free group.

The addition of 0.1 mg% of inert strontium produced markedly lower ($P<0.01$) uptakes of Ca^{45} than did a level of 0.001 mg % of strontium or the carrier-free group. The bones grown in a medium to which carrier strontium had not been added contained twice the radioactivity of the 0.1 mg% strontium group. In another experiment, 1.0 mg % strontium lowered the Ca^{45} uptake by 80% and indicated that stable strontium can markedly interfere with the calcification of bone when present in large enough quantities.

Discussion. Like the kidney and the digestive tract, bone, in forming its solid phase, has exhibited an over-all discrimination against strontium in favor of calcium. The system used for these experiments was artificial and therefore the results may not quantitatively represent what occurs *in vivo*. There are no reasons, however, to assume that embryonic and post-natal bone differ in their processes of bone crystal formation; thus, the qualitative events depicted can be expected to take place in bone metabolism *in vivo*.

The data in this paper show that strontium is favored in the initial uptake by bone by a ratio of 1.08, but that in the removal processes more strontium is lost than calcium by a factor of 1.2. Using the preceding discrimination factors and measurements showing that 4.78% Ca^{45} and 3.98 Sr^{89} were in the bones, it can be calculated by simultaneous equations that the accretion of Ca^{45} and Sr^{89} was 14.78 and 15.98%, respectively, of the available radioactivity. Major portions, 10.0 and 12.0% of the Ca^{45} and Sr^{89}, were removed during this 9-day period by resorption. The net result of these 2 processes would give an O.R. of 0.83. The bones were estimated to have added 0.015 mg of calcium during the 9-day period. From this it was determined that the accretion rate for calcium was 0.0145 mg calcium/hour/mg bone calcium and for resorption was 0.010. In 3-month-old rats, the corresponding rates for the tibias were 0.0026 and 0.0020(6). The values for the embryonic chick bones were about 5 times higher than those for 3-month-old rats. Since the rates do diminish with age, the *in vitro* data presented are in reasonable agreement with that seen *in vivo*.

The addition of large amounts of carrier strontium had very significant effects upon the extent of calcification of bone. This had been encountered before in both *in vitro* and *in vivo* studies(3,7). The addition of 0.1 mg % strontium increased the number of equivalents of calcium plus strontium in the medium by only 0.4% while the amount of Ca^{45} deposited in bone decreased 53%. This would rule out the possibility that strontium acted by competing with calcium for sites of deposition.

Summary. An *in vitro* culturing technic was used in comparing metabolism of calcium-45 and strontium-89 in bone. The results of a large number of determinations indicated: a) that at 2 hrs bone contained 1.08 times as much strontium-89 as calcium-45; b) when exposed to constant levels of Sr^{89} and Ca^{45}, the Observed $Ratio_{bone-medium}$ equilibrated at 0.83 within 7 days; c) strontium-89 was released from bone at a ratio of about 1.2 times that of calcium-45; d) addition of carrier strontium markedly inhibited the long term deposition of calcium in bone but the discriminatory mechanisms were unaltered.

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Toxicity of Beta-Aminopropionitrile for Turkey Poults.* (22857)

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Diets containing *Lathyrus odoratus* seeds or beta-aminopropionitrile (BAPN) cause extreme abnormalities of the skeleton, hernias, dissecting aneurysms of the aorta and reproductive failure in rats(1-4). A striking resemblance was noted between the degenerative changes of the aortas of rats described by Bachhuber and Lalich(4) and the changes observed by McSherry *et al.*(5) in aortas of commercially reared turkeys which died of dissecting aneurysms. This observation prompted an investigation of the possibility of a connection between BAPN, the active fragment of the toxin occurring in *Lathyrus odoratus*, and the field cases of dissecting aneurysms of turkeys.

Methods. Broad Breasted Bronze turkey poults were started in electrically heated batteries with raised wire floors and survivors were transferred to growing batteries with wire floors at approximately 4 weeks of age. Experimental diets were supplied *ad libitum* from 1 day of age. Inasmuch as the condition reported by McSherry *et al.*(5) occurred most frequently in rapidly growing birds consuming high protein rations, a high energy diet calculated to contain 28% protein was used (Table I). The birds were weighed at weekly intervals and the severity of symptoms was recorded at each weighing. In Exp. 77 the birds were scored for severity of leg deformities with a score from 0 for normal to 4 for severe deformities in both legs. Birds which died during the course of the experiment were examined grossly and microscopically for tissue changes. The BAPN used was prepared by addition of ammonia to acrylonitrile and was supplied by Abbott Laboratories.‡ For convenience in storage and incorporation into the experimental diet, the BAPN was converted to the hydrochloride (BAPN • HCl) which is a stable crystalline solid.

TABLE I. Basal Ration.

	g/kg
Ground yellow corn	305
Soybean oil meal	325
Fish meal	100
Oat groats	50
Alfalfa meal	50
Meat scraps	50
Choice white grease	50
Dried whey	50
Chick grit	10
Chick size oyster shell	5
Salt	5
Choline chloride	1
Vit. D_3 supplement (1500 ICU/g)	.9
DL-methionine	.2
B_{12} supplement (6 mg/lb)	.25
Penicillin supplement (4 g/lb)	.5
$MnSO_4 \cdot H_2O$	.33
Plus the following/kg:	
Vit. A palmitate	6,000 USP units
Riboflavin	6 mg
Niacin	40
Ca pantothenate	10
Alpha tocopherol acetate	20

Results. When BAPN • HCl was fed at the rate of 0.25% of the diet, a level on which rats can survive for several weeks, all poults were paralyzed by 1 week of age. Growth was markedly reduced (Table II) and death resulted in less than 2 weeks. Microscopic examination revealed degeneration of the anterior motor neurons (Table III). When

TABLE II. Effect of BAPN • HCl on Growth of Turkey Poults.

Exp.	% BAPN • HCl	Weekly wt in g 0	1	2	3	4	5
67	.0	52	121	267			
	.25	59	81				
69	.0	63	146	272			
	.125	60	117	130			
70	.0	61	116	226	357		
	.0625	66	110	203	307		
72	.0	59	123	274	460	766	1126
	.04	60	122	217	342	635	
	.0313	60	110	246	397	575	743

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‡ Valuable information and materials furnished by Abbott Laboratories are gratefully acknowledged.

TABLE III. Symptoms of BAPN • HCl Toxicity for Turkey Poults.

Exp.	No. birds per group	% BAPN • HCl	Symptoms and lesions
67	11	.25	Paralyzed by 1 wk. Degeneration of anterior motor neurons.
69	6	.125	Paralyzed by 1 wk. 67% died of pericardial hemorrhage by 2 wk.
70	12	.0625	50% hock and toe deformities. 33% died of pericardial hemorrhage and 33% of aortic rupture by 3 wk.
72	12	.04	50% hock and toe deformities by 5 wk. 17% died of pericardial or pulmonary hemorrhage and 50% of aortic rupture.
72	13	.0313	92% hock and toe deformities in 5 wk. 15% died of pericardial or pulmonary hemorrhage and 15% of aortic rupture.

BAPN • HCl was reduced to 0.125% of the diet all birds were paralyzed by 1 week and 67% died of pericardial hemorrhage by 2 weeks. Growth was reduced at this level also. At 0.0625% growth was retarded, 33% of the birds died of pericardial hemorrhage and 33% of aortic rupture in 3 weeks and hock and toe deformities occurred in 50% of the birds. Of birds receiving 0.04% BAPN • HCl, 17% died of pericardial or pulmonary hemorrhage and 50% died of aortic rupture.

The aortae usually ruptured somewhere between the diaphragm and renal arteries. Massive retroperitoneal or peritoneal hemorrhages were most common. In some instances the hematoma would dissect along the aorta and extend into the thorax. A more detailed analysis of the histological changes characteristic of the arterial damage will be published elsewhere.

FIG. 1. Enlarged hock and bent tarso-metatarsus of bird receiving 0.0313% BAPN.

FIG. 2. Illustration of curled toes caused by 0.0313% BAPN.

Fifty percent of birds receiving 0.0313% BAPN • HCl survived to 5 weeks at which time they were transferred to the basal diet to see if recovery were possible. All surviving birds had hock deformities and crooked toes of varying degrees of severity at 5 weeks and only 1 of 13 birds failed to develop such deformities. This bird died prior to 2 weeks of

FIG. 3. Normal bird (left) compared with bird consuming 0.0313% BAPN (right).

age from pericardial hemorrhage. By 5 weeks 15% of the birds had died of pericardial or pulmonary hemorrhage and 15% died of aortic rupture. There was little if any recovery in the crippled birds when maintained from the 5th to 8th week on the basal diet. Figs. 1-3 illustrate the hock and toe deformities of birds receiving 0.0313% BAPN • HCl. The leg bones of some of the more severely deformed birds were removed for comparison to the leg bones of a normal bird. There was slight bending in some of the tarsometatarsi but very little if any of the other abnormalities observed in the skeletons of rats. It would appear that the extreme abnormality of the hock joint involves the ligaments and tendons rather than the bone.

The results of Exp. 77 are shown in Table IV. Growth was depressed by .03, .02 and .01% of BAPN • HCl although only slightly by the lowest level. Leg deformity scores of all groups receiving BAPN were much greater than controls although the scores of birds receiving 0.02% and 0.01% were somewhat less than the birds receiving 0.03%. When the 0.02% added DL methionine of the basal diet was omitted growth was slightly reduced but there appeared to be no effect on degree of deformity. The addition of 100 μg/kg of vit. B_{12} appeared to have no effect on the leg deformity; a high level of all B vitamins appeared to afford some protection against leg deformity but did not improve growth.

Discussion. Beta-aminopropionitrile in the diet of turkey poults at levels as low as 0.01% of the diet caused growth retardation and leg deformities. Also the occurrence of dissecting aneurysms of the aorta and other internal hemorrhages in poults less than 3 weeks of age with diets containing 0.0313% of BAPN • HCl indicates this species is much more susceptible to the toxin of *L. odoratus* than is the weanling rat. The work of Chang *et al.*(6) with *Xenopus larvae,* chick embryos and regenerating newt legs and the investigation of rat embryo resorption by Walker and Wirtschafter(3) indicate that age may be an important factor. The turkey poults in these experiments were one day old while the weanling rats in other experiments were approximately 21 days of age when normally started on experiment. Geiger *et al.*(1) were the first to recognize that age had an effect on degree of deformity of rats consuming *Lathyrus* seeds. Another factor which may contribute to the greater susceptibility of the turkey is its faster growth rate and greater feed con-

TABLE IV. Effect of BAPN • HCl on Growth and Leg Deformity—Exp. 77.

Alterations of basal diet	Weekly wt in g					
	0	1	2	3	4	5
None	53	111	213 (0.1)	362 (0.2)	597 (0.2)	930 (0.3)
+ .03% BAPN • HCl	54	97	187 (0.3)	327 (1.2)	563 (2.0)	836 (2.7)
+ .02% "	53	106	204 (0.9)	340 (1.1)	555 (1.6)	830 (1.9)
+ .01% "	54	104	206 (0.5)	354 (1.0)	584 (1.6)	881 (1.9)
+ .03% " + vit. B_{12}*	54	109	207 (0.5)	365 (1.3)	604 (2.3)	907 (2.2)
Omit DL-methionine	53	107	200 (0)	333 (0.2)	567 (0.2)	872 (0.1)
Omit DL-meth. + .03% BAPN • HCl	52	106	189 (0.5)	318 (0.9)	527 (2.2)	802 (2.3)
+ .03% BAPN • HCl + B vitamins†	52	107	203 (0.5)	346 (0.9)	554 (1.0)	844 (1.1)

* 100 μg/kg of diet.

† The following in mg/kg diet: Vit. B_{12} 0.16, biotin 1.6, menadione 4, pyridoxine 32, pteroylglutamic acid 32, riboflavin 48, Ca pantothenate 160, thiamin 80, niacin 400, para-amino benzoic acid 800.

Figures in parentheses represent avg leg deformity score of group based on a rating of 0-4 for severity.

sumption. The difference in symptoms and lesions between turkeys and rats, however, indicates that there is some species difference not associated with age or feed consumption.

The turkey poult is highly susceptible to hock disorders as indicated by investigations of Scott(7), Hunt *et al.*(8) and Slinger *et al.* (9). While these researches showed that supplemental niacin, vit. E and phosphorus can prevent enlarged hock disorder under certain conditions it is apparent from the last two reports that the problem is a complex one. Hock disorder of uncertain etiology appears in many commercial flocks but usually in only a few birds.

While the turkey poult consuming low levels of BAPN develops abnormalities similar to those which appear under field conditions, it is impossible to say whether or not BAPN is a causative agent in field cases. If BAPN is involved, its source under practical conditions is yet to be located.

Summary. (1) Beta aminopropionitrile hydrochloride was toxic to turkey poults at levels as low as 0.01% of diet. (2) Paralysis, degeneration of anterior motor neurons and growth depression were the chief abnormalities observed at 0.25% and 0.125% while pericardial and pulmonary hemorrhage, ruptured aortas, leg and toe deformities, and growth depression were the primary symptoms at lower levels. (3) Preliminary attempts to alter severity of symptoms by reducing the methionine level or by adding vit. B_{12} or a high level of all B vitamins were not very successful, but further investigation of the B vitamins appears to be justified.

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Lipid Distribution and Metabolism in Two Areas of Aorta of Normal and Cholesterol-fed Rabbits. (22858)

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When cholesterol-fed rabbits are killed at successive periods gross, discrete atherosclerotic lesions appear first in the aortic arch and only much later is the rest of the aorta similarly affected. We have found a high incidence of atherosclerosis in the aortic arch only in rabbits on a high cholesterol diet for 60-70 days but rarely were lesions seen in aorta of rabbits only 40 days on the diet. Duff and McMillan(1) reviewed evidence indicating an importance of "local factors that operate in the vessel wall to determine the localization and to influence the subsequent development of individual atherosclerotic lesions." With due cognizance of the element of time as a factor in the development of atheromatosis, these observations raise the question of the nature of the factor(s) responsible for the localization and site of appearance of the earliest atherosclerotic lesions in the rabbits. The foregoing prompted us to study the distribution of lipids and phospholipid metabolism in 2 areas of the aorta, namely, the arch and the descending limb of the aorta of rabbits on the usual laboratory diet and rabbits kept for 40 and 70 days on a

TABLE I. Lipid Distribution and Specific Activities of Phospholipid in Aortic Arch and the Descending Aorta of Control and Cholesterol-Fed Rabbits for 2 Time-Periods.

Item	Aortic arch			Descending aorta			"P" value of diff. between aortic sections		
	Diet	Cholesterol-suppl. diet*		Diet	Cholesterol-suppl. diet*				
	a. Controls	b. 40 days	c. 70 days	a. Controls	b. 40 days	c. 70 days	a.	b.	c.
Part A	(15)	(5)	(6)	(15)	(5)	(6)			
Total lipid	10.16±.72	10.99 ±1.81	9.31 ±1.01	6.93±.76	5.96±.54	5.38±.91	‡	‡	‡
Phospholipid	.91±.05	.81 ± .09	.88 ± .13	.83±.04	.62±.07†	.74±.05	ns	ns	ns
Total chol.	.18±.02	.18 ± .03	.63 ± .22†(†)	.15±.01	.16±.01	.26±.04†	ns	ns	ns
Free "	.13±.01	.10 ± .04	.33 ± .25	.12±.01	.13±.01	.19±.01‡(‡)	ns	ns	ns
Ester "	.05±.01	.08 ± .01	.30 ± .14	.03±.006	.03±.01	.08±.03	ns	‡	ns
Neutral fat	9.03±.74	9.94 ±1.81	7.57 ±1.12	5.93±.75	5.14±.75	4.32±.83	‡	‡	‡
Part B	(12)	(4)	(4)	(12)	(4)	(4)			
S.A. $\times 10^3$	2.19±.18	2.77 ± .28	3.08 ± .34†	3.15±.31	3.62±.43	2.85±.67	†	ns	ns

* See text for description. Atheromata in aortic arch only of rabbits 70 days on high cholesterol diet; no atherosclerosis in group fed 40 days. All values are mean ± S.E. Figures in parentheses are No. of animals in each group. S.A. (specific activity) is % of inj. P^{32}/mg lipid phosphorus.

Significant differences between means of controls and each exp. group are indicated as follows: † "P" value = <.05 >.01. ‡ "P" values of 0.01 or less. Significant differences between means of two cholesterol-fed groups are indicated by "P" value in parenthesis.

high cholesterol diet. To allow an assessment of the relative status of lipid metabolism in the groups studied, lipid distribution and phospholipid metabolism of plasma and liver also were determined in these animals.

Materials and methods. New Zealand white female rabbits were used. At 10 a.m. and 4 p.m. each day of the experiment a rabbit pellet food (70 g), tap water and 10 g of shredded carrots were given to half the total number of rabbits under investigation while the remaining number of animals were given the same provisions except that the carrot portion was thoroughly mixed with 1 g of cholesterol (crystalline cholesterin, U.S.P., Merck*). This dietary regime was readily and entirely consumed by the rabbits each day for 40 and 70 days. Animals fed the normal (group *a*) and the high-cholesterol diet for the 2 periods (groups *b* and *c*) were killed on the same day; tissues for chemical and radioactivity analyses were obtained within a 10-day period from animals of all groups. Some rabbits of each group received by vein approximately 0.5 mc of radioactive phosphate (P^{32}) on the final day and killed 6 hours later. A large sample of heart blood was obtained and the animal then given an overdose of EVIPAL (n-methyl-cyclo-hexenyl-methyl-barbituric acid). The whole liver and aorta were removed; and the latter then cleaned of adherent tissue. The aorta then was divided into 2 sections: (a) the aortic arch—3.0 cm of vessel from the aortic ring; and (b) the remainder of the aorta. The 2 aortic sections were graded separately by visual judgment for severity of atherosclerosis from normal (zero) to most severe (4 plus). Chemical analyses of tissue lipid fractions and radioactivity (P^{32}) measurements of tissue phospholipid- and acid soluble-phosphorus were done by procedures previously reported(2) from this laboratory. Total lipid was calculated from weight of residue after evaporation of petroleum ether extract and drying to constant weight; lipid and acid soluble phosphorus by the methods of Youngsburg and Youngsburg(3) and Fiske and SubbaRow(4); cholesterol by the method of Schoenheimer and Sperry(5); and neutral fat was calculated by subtracting the sum of the values for phospholipid, free cholesterol and cholesterol oleate from the total lipid value. Measurements of radioactivity were done on duplicate samples of suitable aliquots for a minimum of 4000 counts employing a Tracerlab Autoscaler with

* We wish to thank Frederick K. Heath, for generous supply of cholesterol (Cholesterin, U.S.P., Merck).

TABLE II. Lipids Distribution in Plasma and Liver and Phospholipid Synthesis in Rabbits on a High Cholesterol Diet for 2 Time-Periods.

Item	Diet a. Controls	Cholesterol-suppl. diet* b. 40 days	Cholesterol-suppl. diet* c. 70 days	Diet a. Controls	Cholesterol-suppl. diet* b. 40 days	Cholesterol-suppl. diet* c. 70 days
Part A	Plasma lipids (mg/100 cc)			Liver lipids (g/100 g of tissue)		
	(15)	(5)	(6)	(15)	(5)	(6)
Total lipid	341.0±17.7	1656.0±385.3§	2448.0±553.2§	4.82±.11	5.52±.36	6.82±.49
Phospholipid	124.2± 8.9	319.6± 39.3§	453.2±103.2§	4.01±.14	3.90±.16	4.21±.14
Total chol.	82.3± 5.7	797.3±231.8§	1154.2±214.6§	.28±.007	.65±.13§	1.39±.28§(‡)
Free "	27.3± 1.6	206.9± 66.3‡	322.1± 55.6§	.24±.006	.33±.03§	.41±.06§
Ester "	55.0± 4.2	590.5±196.6‡	832.1±159.8§	.04±.006	.32±.11‡	.98±.23§(‡)
Neutral fat	94.7± 9.2	125.2± 60.7	232.9± 54.8‡	.51±.05	.78±.08‡	.57±.14
Part B	Summarized radioactivity data of plasma and liver phospholipid					
	Plasma phospholipid-phosphorus			Liver phospholipid-phosphorus		
	(12)	(4)	(4)	(12)	(4)	(4)
% I.D.†	10.89±1.01	26.43±1.85 §	32.10±3.15 §	10.11±1.58	12.09±1.83	20.36±3.97‡(‡)
S.A. $\times 10^3$	2.42± .12	2.24± .28	2.22± .73	6.43± .36	8.00±1.11	12.23±2.83‡

* See text for description. Atherosclerosis found in aortic arch only in rabbits fed cholesterol diet 70 days; no atheromata in rabbits fed 40 days high cholesterol diet.

† % of inj. P^{32} incorporated in phospholipid, disintegrations/min.; for plasma expressed/cc $\times 10^5$ and for liver expressed/g $\times 10^3$.

Significant differences between means of control and exp. groups shown as follows:
‡ "P" value = <.05 >.01. § "P" value = <.01. Significant difference between two groups fed cholesterol diet indicated by "P" value shown in parenthesis.

S.A. (specific activity) is % of inj. P^{32}/mg lipid phosphorus. All values are mean ± S.E. Figures in parentheses indicate No. of animals in each group.

a Victoreen No. 1B85 Geiger tube.

Results. The lipid distribution and phospholipid specific activities of the arch and descending limb of aorta of normal and cholesterol-fed groups are presented in Table I. The mean weights of the two areas of aorta from the different groups of rabbits were similar. The aortic arch of normal-diet rabbits weighed .235 g (range .190-.310 g) and that of groups *b* and *c* weighed .214 g and .252 g, respectively. The descending limb of the aorta of normal-diet rabbits weighed .318 g (range .258-.420 g) and that of the 2 cholesterol-fed groups weighed .332 g and .376 g, respectively. In agreement with the signs of gross atherosclerosis detected in the 3 groups of animals, cholesterol concentrations were significantly elevated in the 2 aortic areas only in rabbits on the high-cholesterol diet for 70 days.

The results of special interest with regard to aortic lipid constitution may be seen from the comparisons of the concentrations of the different lipid fractions of the arch and the descending limb of the aorta in the same group of rabbits. The results show that total lipid and neutral fat concentrations in the descending limb of aorta were significantly less than that of the aortic arch in each of the groups. Further, the radioactivity data indicate that the rates of phospholipid synthesis in the 2 areas of aorta were significantly different in the normal-diet rabbits. However, this was not the case for the groups on the high-cholesterol diet. Specific activities of phospholipid of the aortic arch and the descending limb of aorta were similar for both cholesterol-fed groups. It may be seen that the latter findings were due to an increased rate of synthesis of phospholipid in the aortic arch, particularly in the rabbits of group *c*. This kind of change in aortic phospholipid metabolism was reported(6,7) to be associated with cholesterol deposition and the development of overt atherosclerosis. Thus, the distinction in rates of synthesis of phospholipid apparent in the aortic arch and descending limb of aorta of normal rabbits was eliminated with the development of gross atheromatous lesions in the longer-term cholesterol-fed rabbits.

The chemical and radioactivity data of

TABLE III. Plasma Lipids Relationships of Control and Cholesterol-Fed Rabbits for 2 Time-Periods.

Ratios	Diet a. Controls	Cholesterol-suppl. diet* b. 40 days	Cholesterol-suppl. diet* c. 70 days
	(15)	(5)	(6)
Free cholesterol/Total cholesterol	.34 ± .009	.26 ± .004§	.28 ± .008§(‡)
Total cholesterol/Phospholipid	.70 ± .05	2.34 ± .58 §	3.02 ± .59 §
Phospholipid/Neutral fat	1.49 ± .18	2.33 ± .79	2.63 ± .78
Total cholesterol/Neutral fat	1.00 ± .13	4.64 ±1.17 §	5.92 ±1.11 §

*‡§ See legend of Table II.

plasma and liver and plasma lipid relationships of the 3 groups of rabbits are presented in Tables II and III. Plasma cholesterol concentrations and phospholipid levels of groups *b* and *c* were not statistically different, but these lipids were 8 and 4 times greater, respectively, than the plasma levels of normal-diet rabbits. Further, the data presented in Table III show that plasma lipid relationships were not different in both cholesterol-fed groups with the exception of the ratio of free cholesterol/total cholesterol, while in all instances but one the ratios of plasma lipid fractions of both cholesterol-fed groups were markedly different from the plasma lipid relationships of the rabbits on the normal diet. The radioactivity data also show that plasma phospholipid synthesis was accelerated to a similar extent in rabbits 40 and 70 days on the cholesterol diet and in both groups the rate of formation of this lipid was considerably greater than that of the controls. On the other hand, the chemical and radioactivity analyses of liver lipid constitution show that total and ester cholesterol concentration and liver phospholipid synthesis were significantly greater in rabbits of group *c* than those on the cholesterol-supplemented diet for only 40 days.

Discussion. The 2 groups of rabbits on the cholesterol-supplemented diet were distinguished from each other with respect to the duration of the regime and also with regard to the presence of atherosclerosis. Only one rabbit of group *b* had grossly visible plaques in the aortic arch; this animal was discarded from the group for presentation of the data. On the other hand, every rabbit fed the high-cholesterol diet for 70 days presented gross lesions in the aortic arch only which were graded as 2-4 plus in severity. Aorta of rabbits on the regular laboratory food pellets alone were entirely free of gross signs of atherosclerosis.

Our findings show that the hypercholesterolemia, altered plasma lipid relationships and increased synthesis of plasma phospholipid in rabbits after 40 days on a high-cholesterol diet were not essentially different from that of rabbits on the same diet for 70 days and, in fact, were the same as the results obtained in rabbits on this dietary regime for 4 months (8). Wang *et al.*(9) found in cholesterol-fed rabbits a steady rise of blood cholesterol level until the 40th day of the diet and then a relatively maintained level of this lipid for the next 4 months. Since aortic atherosclerosis in cholesterol-fed rabbits is rarely evident before 60 days of this dietary regime, the evidence indicates that conditions favoring the development of atherosclerosis may exist for some time before the eventual evolution of gross lesions.

One implication of the time-lapse in the development of atherosclerosis is that factors (? metabolic) in the aortic wall are significantly related to the deposition of lipids in this tissue. It is known that in the cholesterol-fed rabbit discrete, local lesions develop first in the ascending limb of the aortic arch and these later enlarge, appear to coalesce, and progress down the aorta. In discussing these morphologic features, Duff and McMillan(1) suggest that local factors "operate in the vessel wall to determine the localization and to influence the subsequent development of individual atherosclerotic lesions." In this study evidence was presented that the neutral fat content of the aortic arch of normal rabbits was different from that of the remainder

of the aorta and the rate of phospholipid synthesis in the arch was significantly less than that of the descending limb of the aorta. It is of interest that Werthessen *et al.*(10) reported a concentration gradient of cholesterol over the length of the calf's aorta. Lower concentrations of cholesterol were found in the arch than at more distal sections of the aorta. Although these facts suggest some fundamental metabolic differences between certain areas of the aorta, their possible relationship to the pathogenesis of atherosclerosis must await further studies of arterial metabolism.

Summary. Lipid partition and phospholipid metabolism of the aortic arch and descending limb of aorta, plasma and liver were determined in groups of rabbits on a normal diet and 40 and 70 days on a cholesterol-supplemented diet. In all groups, the descending limb of aorta had significantly lower total lipid, primarily due to decreased neutral fat concentration, compared with the aortic arch. In normal rabbits the specific activity of phospholipid in the descending aorta was significantly greater than that of the aortic arch. However, this distinction in phospholipid synthesis between the two areas of aorta was not found in the groups of rabbits on the high-cholesterol diet. The radioactivity data indicated this was due to an increased rate of formation of phospholipid in the aortic arch of rabbits fed the cholesterol-supplemented diet, particularly those rabbits on the diet for 70 days, and probably was related to the development of atheromatous lesions in the arch only of these rabbits. The results were discussed in relation to the general problem of local factors in the arterial wall influencing the localization and subsequent development of atherosclerotic lesions.

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Induced Resistance in Mice to Intravenous Toxicity of Influenza Virus.* (22859)

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Protection of intact animals from rapidly appearing damage, usually referred to as toxicity, which follows inoculation with massive doses of influenza viruses has been reported (1-5), but attention has largely been directed to toxicity following intracerebral injection in mice, to lung consolidation caused by nasal installation of certain strains into mice, and to the pyrogenic effect of intravenous injection into rabbits. Beyond the initial description of intravenous toxicity of influenza virus in mice(6), little study has been applied to this phenomenon. When it was found, during studies of factors modifying host reactions to

* Opinions expressed in this article are those of the authors and do not necessarily reflect the views of the Navy Department.

† Some data in this report were included in thesis submitted to Graduate School of University of Wisconsin in partial fulfillment of requirements for degree of Doctor of Philosophy. Present address: Indiana University Medical Center, Indianapolis.

virus infections, that mice could be protected from lethal effects of large intravenous doses of influenza virus by preceding sub-lethal injections of virus, it appeared desirable to determine in what respect this protection corresponded to that observed in the other systems and to explore further the relationships of viral species, dose, and injection intervals that would produce increased resistance.

Materials and methods. The PR-8 strain of influenza A virus, Lee strain of influenza B virus, Enders strain of mumps virus, and GB and (H) Najarian strains of Newcastle disease virus (NDV) were cultivated in the allantoic sac of embryonated eggs and used as infected, undiluted allantoic fluid or diluted in phosphate buffered saline. The number of agglutinating doses (A.D.) in these preparations was determined by the pattern technic using a final concentration of 0.5% chicken erythrocytes. The Chicago strain of feline pneumonitis and Madrid E strain of *R. prowazeki* were cultivated in the yolk sac of embryonated eggs and used as yolk sac suspensions clarified by light centrifugation and diluted in Hanks' balanced salt solution. Receptor destroying enzyme (RDE) was purified by the method of Ada and French (7) from filtrates of cultures of *Vibrio cholerae* (Inabá), Culture 35A3 of the National Institutes of Health. This preparation had a titer of 1:1024 as measured by the method of Burnet(8). Swiss white mice (Webster strain, mixed sexes) weighing 10-14 g and 4-5 weeks old were used. Pre-challenge injections were in volumes of 0.25 or 0.5 ml. For challenge injections and toxicity titrations virus suspensions were injected into a tail vein in 1.0 ml volume and only deaths occurring within 96 hours were considered due to toxic effects of virus. Using six mice per group and two-fold dilutions of virus, it was found in ten complete titrations of one virus preparation that the standard deviation of the distribution of the LD_{50} end points was 0.220 $\log_{10}$ units. It could be calculated from this that the chances were 19 out of 20 that a four-fold difference between the results of two titrations was significant.

TABLE I. Protective Effect of Intravenous Injections of Active and Heated PR-8 Virus against Homologous Challenge 24 Hr Later.

Pre-challenge inj.	Challenge LD_{50} (A.D.)
Normal allantoic fluid	422
Active virus—96 A.D.	5120
56° heated virus—1280 A.D.*	1808
70° " " *	640

* A.D. before heating = 5120.

Results. Protective effect of sub-lethal doses of homologous virus. Groups of mice were given intravenous injections of homologous PR-8 virus, either as a small sub-lethal dose (¼ i.v. LD_{50}) of fully active virus or as a much larger quantity of heated virus. These mice were challenged 24 hours later with intravenous injection of fully active, toxic virus. It was found (Table I) that sub-lethal doses of active, homologous virus increased the LD_{50} at challenge by approximately 12-fold. There was not a complete refractoriness for if the challenge inoculum was large enough the mice died. Homologous virus heated at 56°C for ½ hour and without detectable infectivity but still retaining hemagglutinating capacity was not completely devoid of protective activity but was less effective than live virus. Virus heated at 70°C for ½ hr and lacking in hemagglutinating activity as well as infectivity did not produce significant protection even when administered in massive quantities. It is to be noted in Table I that the preparatory dose of heated virus was many times larger than the preparatory dose of active virus.

Heterologous virus. Other viruses of the mumps - influenza - Newcastle disease virus group serologically unrelated to the PR-8 strain of influenza A were similarly tested for their capacity to protect mice against the intravenous toxicity of this strain. Preliminary titration showed that each of two strains of Newcastle disease virus caused 50% mortality in mice on intravenous injection of approximately 1280 A.D. of active virus as did the Lee strain of influenza B. Fully active mumps virus in quantities available in undiluted allantoic fluid without concentration (640 A.D./cc) did not produce apparent deleterious effects. Intravenous injections of

TABLE II. Protective Effect of Intravenous Injections of Heterologous Virus Given 24 Hr before Intravenous PR-8 Challenge.

Pre-challenge inj.*	Challenge LD_{50} (A.D.)
Normal allantoic fluid	269
Active Lee	2560
70° heated Lee	422
Active mumps	2560
70° heated mumps	380
Active NDV-(H) Najarian strain	2560
" " GB strain	2560
70° heated NDV-GB	422

* Active viruses = 320 A.D.

320 A.D. of each of these viruses given 24 hours before challenge with PR-8 virus increased the LD_{50} by more than 9-fold (Table II) but injection of these preparations after exposure to sufficient heat to destroy detectable viral infectivity and hemagglutinin was without protective effect. Other experiments demonstrated that a similar result followed injection of the virus strains in reverse order, *i.e.*, if the Lee strain of influenza B was employed as the toxic challenge, pre-challenge injections of sub-lethal quantities of the PR-8 strain of influenza A served effectively to protect mice.

With the demonstration of the protective effect of several viruses of the mumps-NDV-influenza group experiments were designed to determine whether pre-challenge inoculation of unrelated viruses or a non-viral pyrogen would alter susceptibility to influenza toxicity. Feline pneumonitis virus in preparations of relatively high concentration, the Madrid E strain of epidemic typhus, and a typhoid vaccine were used. The feline pneumonitis virus and the typhus rickettsiae were titrated for intravenous toxicity in mice and doses of ¼-½ LD_{50} of fresh, live suspensions were used as preliminary inoculum. A commercial typhoid vaccine was diluted 1:4 in physiologic saline and 0.25 ml aliquots containing 6 x 10^7 *S. typhosa*, 1.6 x 10^7 *S. paratyphi* A, and 1.6 x 10^7 *S. paratyphi* B were injected intravenously into mice. There was no evidence of significant protection of mice by any of these three agents against challenge 24 hours later with 3 LD_{50} of PR-8 virus.

Minimal protective dose of homologous and heterologous virus. Time of onset and duration of resistance. The quantity of live virus required to protect against a standard intravenous challenge of 6 LD_{50} of PR-8 virus given 24 hours later was determined by varying the pre-challenge inoculum in serial 2 or 4-fold steps. All mice were protected by 20 A.D. of homologous PR-8 virus, by 20 A.D. of mumps virus, and by 20-40 A.D. of the GB strain of NDV. Smaller doses conferred diminishing protection and the gradation from complete protection to complete susceptibility was spread over 3-5 two-fold steps.

Time required for development of the resistant state and duration of resistance after injection of small, constant quantities of live mumps virus or NDV was determined using 40 A.D. of NDV or mumps virus as the pre-challenge inoculum and 6 LD_{50} of PR-8 virus as a challenge. It was found (Table III) that by 13 hours after injection of NDV all mice survived challenge with PR-8 virus and that this refractoriness persisted unabated through the 48th hour but had waned somewhat by 72 hours. With mumps virus as the preliminary inoculum, however, resistance did not become complete for 30 hours. Considerable resistance persisted through 72 hours but was subsiding at 96 hours.

Protective effect of receptor destroying enzyme. It was found that intravenous injection of 0.5 ml of purified RDE 24 hours be-

TABLE III. Time of Onset and Duration of Resistance to Intravenous PR-8 Toxicity after Pre-Challenge Injection of NDV or Mumps Virus (40 A.D.).

	Hr to challenge*									
Pre-challenge inj.	3	6	10	13	18	24	30	48	72	96
NDV	6/8†	5/8		0/8	0/8	0/8	0/8	0/8	5/8	7/8
Mumps	7/8	7/8	6/8	4/8	4/8	1/8	0/8	2/8	2/8	6/8
Normal allantoic fluid	8/8				8/8					

* 6 LD_{50}.

† Numerators give mice dying in 96 hr after challenge; denominators, total mice in group.

TABLE IV. Protective Effect of RDE against Intravenous Toxicity of PR-8 Influenza A Virus.

Pre-challenge inj.*	Challenge Agglut. doses of virus 2560	1280	640	320	160
Saline	5/5†	6/6	4/6	1/6	0/6
Unheated RDE	2/6	0/6	0/6	0/6	0/6
Heated RDE	6/6	2/6	0/6	0/6	0/6

* 0.5 ml intrav. 24 hr before challenge.
† No. of mice dying in 96 hr/total mice in group.

fore challenge completely protected mice against as many as 2560 A.D. (8 LD_{50}) of PR-8 virus although RDE given only 30 minutes after the virus was without any protective effect. The time required for development of this refractory state was tested in a manner similar to that described for sublethal doses of viruses. Mice given RDE intravenously and challenged with 2560 A.D. of PR-8 at intervals from 15 minutes to 4 hours after RDE injection were fully susceptible to the lethal effect of PR-8, but by the next test interval, 8 hours, sufficient resistance had developed so that all mice survived challenge, as they did also at 24 hours after the protective injection. The duration of this resistance was not determined.

To determine whether the protective effect of the RDE preparation was due to its content of RDE or to other unidentified components, the protection conferred on mice by the RDE preparation was compared with that produced by an aliquot of RDE which had been boiled for 30 minutes. This heating resulted in complete destruction of the erythrocyte receptor destroying activity of the RDE preparation as measured in the usual hemagglutination-inhibition test. The results of such an experiment in which mice were given intravenous injections of active and heated RDE and challenged 24 hours later with 4 LD_{50} of PR-8 virus are presented in Table IV. It is evident that the protective effect of the RDE preparation was reduced somewhat by heating but not entirely destroyed.

Discussion. The rapidity with which resistance develops, its temporary nature, and its production by serologically heterologous virus make it quite unlikely that specific immunity based upon antibody production plays any part in the increased resistance in these experiments.

Although RDE produces a refractory state, the fact that much of the resistance inducing potency remains after heat destruction of the erythrocyte receptor destroying capacity suggests that protection by RDE is not dependent upon destruction of mucoprotein cellular receptors unless receptors on the involved tissue cells are much more susceptible to destruction by residual enzyme than are those of erythrocytes. A similar protection of mice by RDE against intracerebral toxicity has been reported(5) but little can be deduced at present as to the mechanism of this effect.

It is of interest that while homologous and heterologous influenza virus, NDV, and mumps virus readily produce a refractory state, feline pneumonitis virus, *R. prowazeki,* and typhoid vaccine had no effect on resistance to influenza virus even though the intravenous toxicity of these latter agents is similar in several respects to that of the mumps, NDV, influenza group of viruses. The refractory state thus exhibits more specificity than that of a tolerance to the endotoxin-like qualities of these agents. In the time lag prior to onset of resistance, the need for much larger quantities of heated than of unheated virus to induce resistance, the transient and incomplete nature of the refractoriness, and the limited range of effective resistance inducing agents the increased resistance to influenza toxicity appears quite similar to the phenomenon of virus interference. Some of these characteristics of the reaction also suggest that some degree of multiplication by the active pre-challenge virus may take place in the affected tissues. Limited multiplication of unadapted influenza virus in mouse brain has been demonstrated(9) and it is conceivable that similar multiplication may occur in other organs. Alteration in the susceptibility of tissue cells brought about by intracellular establishment of the pre-challenge virus would seem to be the most likely explanation of this acquired resistance. It is noteworthy, however, that Wagner(10) was unable to demonstrate an increase in resis-

tance of cells (HeLa) in culture to the cytotoxic action of influenza virus after exposure to subtoxic quantities of virus. It appears that it will be necessary to follow events in the actual cells involved in these toxicity reactions during development of host resistance in order to localize the site of altered susceptibility.

Summary. Sublethal quantities of homologous and heterologous influenza virus, mumps virus, and Newcastle disease virus injected intravenously in mice produced a state of resistance to the toxic effects of large quantities of intravenously injected PR-8 influenza A virus. Virus heated sufficiently to destroy infectivity was less effective in inducing resistance than was fully active virus; destruction of hemagglutinin caused complete loss of resistance inducing activity. Injection of sublethal doses of virus was followed by a lag period, which varied with the virus injected, before resistance became demonstrable, and resistance waned after 3-4 days. No increase in resistance to influenza toxicity was demonstrable after pre-challenge injection of feline pneumonitis virus, *R. prowazeki*, or typhoid vaccine. A preparation of receptor destroying enzyme (RDE) was effective in inducing increased resistance but its effect could not be attributed to its capacity for destruction of mucoprotein receptors since heat destruction of this activity did not completely destroy its resistance inducing effect.

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Intracellular Multiplication of *Brucella abortus* in Normal and Immune Mononuclear Phagocytes.* (22860)

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Importance of mononuclear phagocytes in pathogenesis of brucellosis has been suspected by many, but the role of these cells in resistance to brucella infection has not been investigated. The intimate relationships of mononuclear phagocytic cells and brucella can be studied *in vivo* only with great difficulty. The ease with which fairly pure cultures of guinea pig mononuclear phagocytes can be obtained and maintained *in vitro* for reasonable periods of time offered a means of studying this problem. The technics used in this work allowed the numbers of both phagocytic cells and viable brucellae to be controlled with a fair degree of accuracy. Some of the difficulties inherent in working with varying ratios of bacteria and tissue cells are thus eliminated.

Materials and methods. Tissue culture technics were similar to those described by Leighton and Hanks(1). Peritoneal exudates,

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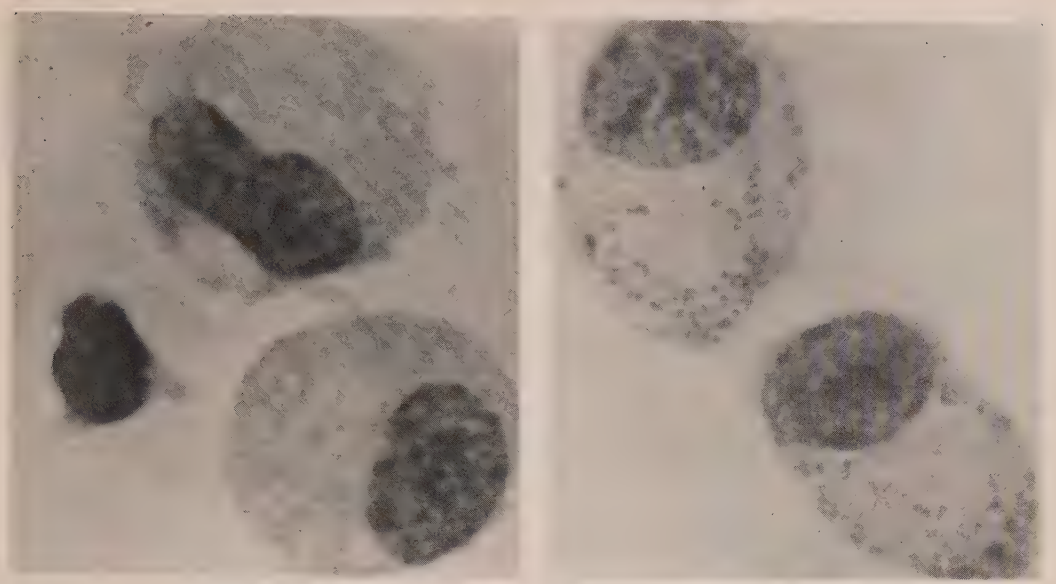
† At present McLaughling fellow at University of Texas Medical Branch in Galveston.



a b

FIG. 1. (a) Normal monocytes from 40 hr cultures containing 10 μg streptomycin/ml but no brucellae. (b) Normal monocytes showing intracellular brucella from 40 hr brucella containing cultures in which supernatant was replaced with sterile cell-free streptomycin containing medium after 2 hr preliminary incubation. May-Grunwald-Giemsa stain. × 2500.

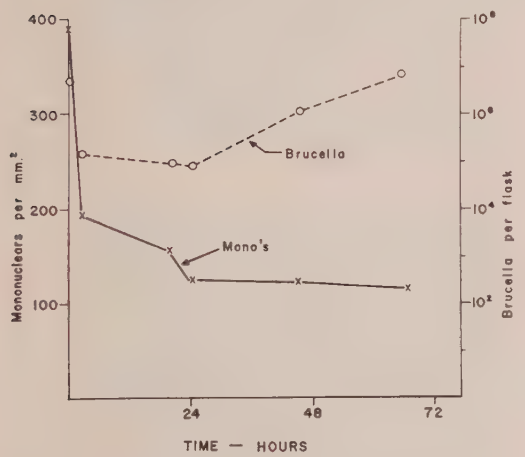


FIG. 2. Shows that there was a definite intracellular multiplication of brucella as evidenced by increase in No. of organisms in cultures while No. of phagocytes remained approximately the same. Note that "0" time shown here is time of beginning of experiment. Medium replaced at 2 hr.

rich in mononuclear phagocytes, were obtained by injection of a dilute solution of glycogen(2,3). Cells were harvested 5 days after injection of glycogen, using heparinized Hanks' solution, centrifuged, washed once with plain Hanks' solution and resuspended in medium composed of 70% Hanks' and 30% normal guinea pig serum to give a concentration of approximately 1 million cells per ml. Living, smooth *Br. abortus* organisms (stain - Lo), from 48-hour-old cultures on tryptose agar, were added to medium in proportion of 50 bacteria per cell. One and a half ml aliquots of mixture were transferred to Porter flasks containing flying coverslips and incubated 2 hours. This time was sufficient for leucocytes to settle and ingest the

TABLE I. Distribution of Intracellular *Br. abortus* among 50 Mononuclear Phagocytes in Stained Coverslip Preparations, at Different Intervals after Replacing Supernatant with Streptomycin Containing Medium.

Hr after replacing medium	Mode (No. of *Brucella abortus* most common in each of 50 phagocytes)
0	1–2
2	1–2
20	7–8
26	11–15
47	25*
68	25*

* This category is arbitrary. Many cells contained brucellae far in excess of 25.

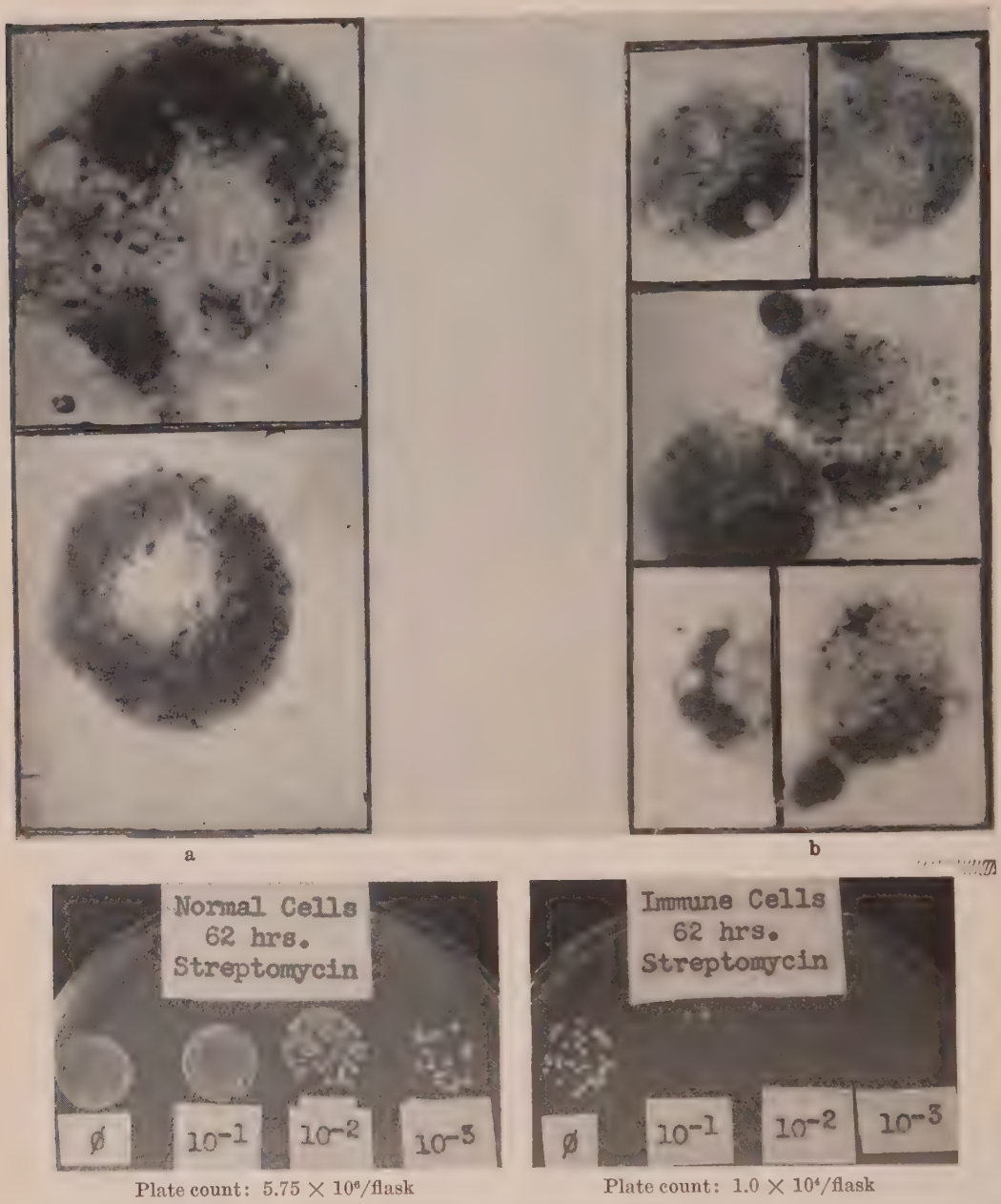


FIG. 3. Showing difference in No. of intracellular brucellae in normal and immune mononuclear phagocytes from 62-hr-old cultures in medium containing streptomycin. (a) *Normal phagocytes* with large compact masses of brucellae and central clear space with few or no organisms. Notice correspondingly high counts in cultures from same flask. b. *Immune cells* with dark cytoplasm (intense diffuse pink in color prints) and few intact organisms. Notice vacuolation of cells and correspondingly low colony count.

bacteria. After this preliminary incubation supernatant was replaced with fresh cell-free medium containing 10 μg of streptomycin per ml. which was enough to kill all extracellular brucellae without producing noticeable effects on the leucocytes or intracellular organisms. The medium was pipetted off at various times and coverslips removed.

stained by Machiavello's technic or Jacobson's modification of May-Grunwald-Giemsa (1-a), and number of intracellular brucellae in each of 50 cells counted. Leucocytes remaining in flask were scraped off with a rubber policeman and washed twice with Hanks' solution. Cells were resuspended in 5 ml Hanks', transferred to special cups, shaken in Mickle tissue disintegrator at optimal amplitude for 2 minutes, and bacterial counts made by modification(4) of surface plate drop method of Miles and Misra(5). Relative counts of mononuclears in flask were made using flying coverslips. The number of cells per mm^2 were counted using an ocular micrometer. These counts gave only relative numbers but these were sufficient to determine the trend. We were interested in whether or not the number of tissue cells were fairly constant during the period of multiplication of the bacteria. This count acts to control such factors as reingestion of bacteria and differential disruption of sensitive cells. The initial drop in numbers of cells (Fig. 2) was seen whether or not the infectious agent was added to the cultures.

Results with normal monocytes. The results of a typical experiment using mononuclear phagocytes from normal guinea pigs are summarized in Figs. 1 and 2 and Table I.

The data show that viable brucellae increased approximately one hundred fold while phagocytic cell count remained almost constant. During the same time the stainable intracellular brucellae also increased markedly. Even with such great increase of intracellular bacteria tissue cells tolerated the organisms fairly well.

Results with immune mononuclear phagocytes. A comparison of findings with normal and immune mononuclear phagocytes is shown in Fig. 3 (a, b) and Tables II and III. We must emphasize the fact that mononuclear cells, obtained from guinea pigs which had been previously inoculated with living brucellae, always remained sterile after one week incubation in Hanks-serum medium and stained preparations from these cultures did not contain intracellular organisms. Intracellular brucellae always derived from or-

TABLE II. Comparison between Intracellular Growth of *Br. abortus* in Normal Guinea Pig Phagocytes (NC) and in Phagocytes Obtained from Guinea Pigs Which Had Been Inoculated with *Br. abortus* 2 Months Previously (IC).

Hr after change of medium	Kind of cells	Colony count/flask	Distribution of brucella among 50 monocytes in stained coverslips preparations									Remarks
			0	1–2	3–4	5–6	7–8	9–10	11–15	16–25	>25	
0	NC	4.75×10^5	22	11	10	5	1	1	0	0	0	About 50% of cells contained small numbers of brucellae. No apparent disintegration of intracellular organisms.
	IC	5.5 "	30	8	7	4	1	0	0	0	0	Same as for normal cells.
20	NC	1.5×10^4	0	3	4	9	5	4	12	5	8	
	IC	7.5×10^2	2	2	4	10	4	6	7	7	8	
40	NC	5.0×10^4	0	3	1	6	6	3	8	5	19	6 of 50 cells loaded with brucella. Few cells showed pale pink color of cytoplasm.
	IC	7.5×10^2	0	5	5	6	7	3	12	6	6	Many cells showed moderate pink coloration of cytoplasm.
62	NC	5.75×10^6	0	2	2	6	5	5	10	4	16	8 of 50 cells counted loaded with brucella. Some slight pink coloration of cytoplasm.
	IC	1.0×10^3	0	5	6	7	3	3	8	4	14	One of 50 cells loaded with brucella. Majority of cells showed marked diffuse pink coloration of cytoplasm.

This experiment was repeated 4 times with similar results.

TABLE III. Comparison between Intracellular Growth of *Br. abortus* in Normal Phagocytes (NC) from Normal Guinea Pigs 173 and 174 and in Immune Phagocytes (IC) from Guinea Pig (No. 125) Which Had Been Inoculated with *Br. abortus* 16 Months Previously.*

Hrs after change to streptomycin containing medium	Cells	No. of colonies of brucellae/flask
28	NC (173)	2.5×10^5
	IC (125)	5.0×10^4
	NC (174)	3.0×10^5
48	" (173)	8.7×10^5
	IC (125)	3.0×10^4
	NC (174)	9.0×10^5
62	" (173)	4.5×10^6
	IC (125)	7.5×10^4
	NC (174)	1.2×10^6

* Preliminary experiments with mononuclear phagocytes from 3 guinea pigs 10 days after inoculation with living brucellae, and from 2 animals 18 mo after infection, gave in each case results comparable to those obtained with cells from normal controls.

ganisms added to the medium. These experiments show that brucellae within immune mononuclear phagocytes do not reach the numbers they do within normal cells. There is good evidence that some destruction of bacilli takes place within the cells and that this results in a pink staining of the cytoplasm presumably due to products of bacterial disintegration.

In preparations stained by Machiavello's method and in color prints, differences between normal and immune phagocytes are prominent. Intact intracellular brucellae and their products of disintegration stain red, while the host cell stains blue.

Discussion. Brucella abortus has long been thought to be capable of intracellular parasitism. This study demonstrates that brucellae can survive phagocytosis and multiply within mononuclear phagocytes of normal guinea pigs maintained *in vitro*. The mononuclear host cells do not show any prominent evidence of injury during a 3-day period of intracellular growth of *Br. abortus*. However, disruption of the cells takes places when the bacilli reach such numbers as to seem capable of mechanically bursting the phagocytes. The observations made during this study do not suggest that any potent toxic material is being released by the bacteria.

Cells obtained from infected (immune) guinea pigs seem capable of either destroying intracellular brucellae after intracellular multiplication has taken place or of suppressing intracellular growth. Most cells were infected with few bacilli during the preliminary incubation period and the outcome must depend on the degree of resistance of the host cell, the virulence of the parasite and the degree of sensitivity of the phagocyte to brucellae and their products. Differences in virulence could be an important factor, as pointed out by Braun,‡ because we are most probably using heterogenous cultures composed of individual cells of different virulence. This may explain why, even after prolonged incubation, there are always some cells which seem to have very few intracellular organisms.

This study indicates that guinea pig mononuclear phagocytic cells and *Brucella abortus* constitute a good model system for the study of host-parasite relationships at the cellular level. In general we have reached similar conclusions as Lurie(6) and Suter(2,3) who worked with *Mycobacterium tuberculosis*. The brucellae, however, offer obvious technical advantages over *Mycobacterium tuberculosis*, in studies of this nature. The brucella-phagocyte system is more amenable to quantitative manipulation.

Summary. The results of typical experiments in host-parasite relationships at cellular level, using *Brucella abortus*-mononuclear phagocyte systems, have been presented. The intracellular multiplication of *Br. abortus* in normal guinea pig mononuclear phagocytes was observed. In *Brucella abortus*-immune mononuclear phagocyte systems the multiplication of the organisms was significantly restricted.

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DNA Synthesis and Incorporation of P^{32} in Irradiated Ehrlich Ascites Cells.* (22861)

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The experiments to be reported are part of a series of studies on the disturbances of desoxyribose nucleic acid (DNA) metabolism after irradiation. The interpretation of biochemical changes observed in irradiated mammalian tissues is complicated by the fact that they are generally composed of mixed cell populations(1). To circumvent this difficulty, many investigators have recently used ascites tumors since they can be obtained as almost pure cell suspensions(2,3,4).

It has been suggested(5,6,7) that while incorporation of precursors into DNA normally occurs only at the time of DNA synthesis, incorporation after irradiation might be due to turnover of existing DNA not accompanied by net synthesis. This seemed an important point which warranted further investigation.

Materials and methods. Ehrlich ascites tumor used was kindly donated by Dr. E. N. Sassenrath and had been carried in C_3H mice for several years. For each experiment 20 to 30 C_3H mice were inoculated intraperitoneally with ascitic fluid containing approximately 2 x 10^7 cells. All measurements were carried out between the fourth and sixth day after transplantation. Irradiated and control groups were always run simultaneously, and a minimum of 5 but frequently 10 mice were used for each point. The total body irradiation (800 r or 1400 r) was carried out as previously described(1). Cell number per mouse for the growth curves was calculated from the total volume of ascitic fluid and cell concentration in the ascitic fluid. To determine the volume of ascitic fluid 2 μc of radioiodinated (I^{131}) serum albumin (RISA, Abbott Laboratories) were injected intraperitoneally after the mouse had been sacrificed. After a 3 minute mixing period, ascitic fluid was withdrawn and counts in a 20 μl aliquot were compared to the injected dose. Cell concentration was determined by counting duplicate dilutions in a hemocytometer. DNA content and total nucleic acid content were determined in duplicate aliquots of ascitic fluid from each mouse. After the cells were centrifuged, their nucleic acids were extracted by the hot perchloric acid method of Ogur and Rosen(8). Total nucleic acid content of the extract was determined by ultraviolet absorption and the DNA content by Ceriotti's indole method(9). For calculation of mean nucleic acid content per cell the concentration of tumor cells was determined by "large cell counts"(10) in a hemocytometer. Since the proportion of non-tumor cells was consistently low (around 5% as determined by enumeration of small cells in the hemocytometer or differential counts on stained smears), no correction was made for their presence. Mean cell volume was calculated from the cell concentration and the "hematocrit" as measured in micro-hematocrit capillary tubes. Separate groups of mice were used for the incorporation studies. They were injected intraperitoneally with 20 μc $Na_2HP^{32}O_4$ at varying times after irradiation together with unirradiated controls and sacrificed 2 hours after injection. Ascitic fluid from 2 mice was pooled, and the cells sepa-

* This work was supported by U. S. Atomic Energy Commission and by Cancer Research Fund of University of California.

TABLE I. Representative Values* from Experiments on Ehrlich Ascites Tumor 4 Days after Transplant.

DNA/cell, pg	Total nucleic acid/cell, pg	Cell vol, μ^3	No. of mice used	No. of determinations
24.9 ± .6	60.7 ± 1.2	2242 ± 61	5	10

DNA specific activity†	DNA specific activity as % inorganic phosphate	DNA specific activity as % acid soluble organic phosphate	No. of mice used	No. of determinations
.272 ± .012	3.54 ± .24	6.05 ± .57	10	5

* Mean values ± stand. error of mean.
† Specific activity expressed as counts/min./mg phosphorus divided by c.p.m. inj./g mouse. Two-hr P^{32} incorporation period.

rated from supernatant fluid. The cells were homogenized in 10% trichloroacetic acid (TCA) for extraction of acid soluble phosphorus compounds. The inorganic phosphate was precipitated from the TCA extract by the method of Le Page(11) and the remaining fraction was designated as the organic acid soluble fraction. DNA was isolated from the original TCA precipitate by a previously published method(12), and specific activities were determined. DNA content of individual nuclei was measured microspectrophotometrically on Feulgen stained smears by a previously described technic(13). Sixty cells were measured in both irradiated and control groups.

Results. Table I lists representative control values for mean DNA content and total nucleic acid content per cell and mean cell volume. Cell volume and total nucleic acid content per cell agree well with values reported by Klein and Forssberg(2), but the DNA content is considerably higher. Both the biochemical determinations and the Feulgen data (Fig. 3) yield a mean DNA content approximately 70% above the theoretical tetraploid DNA value. It is not known whether this high value is due to a large number of cells which are in the process of DNA synthesis or whether it represents a high proportion of hypertetraploid cells in our tumor line.

Table I also lists the DNA specific activity after a 2-hour P^{32} incorporation period, expressed in absolute units and as percent of the specific activity of intracellular inorganic phosphate and acid soluble organic phosphate.

In a few experiments DNA specific activity was determined 2, 4, and 6 hours after administration of P^{32}. When rate of formation of DNA was calculated using the specific activity of acid soluble organic phosphate as the precursor specific activity, the results were somewhat variable but averaged 1.7% per hour.

In most experiments 800 r total body irradiation was administered on the fourth day after transplanting when the cell number per mouse was approximately 3 x 10^8. During the 2 days following irradiation the cell number did not change measurably, while it increased in controls at the rate of 1.5% per

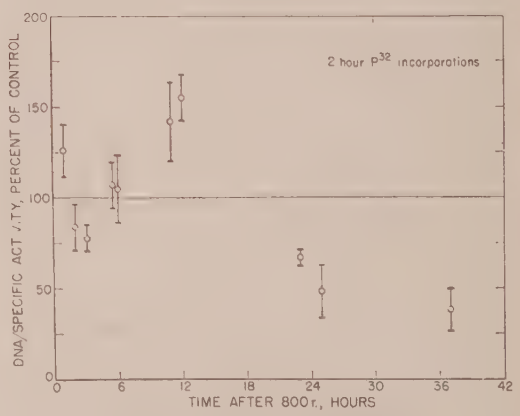

FIG. 1. 2-hr incorporation of P^{32} into DNA at varying times after radiation.*

* Values are mean specific activities as % of control. Vertical lines represent stand. errors of the ratios.

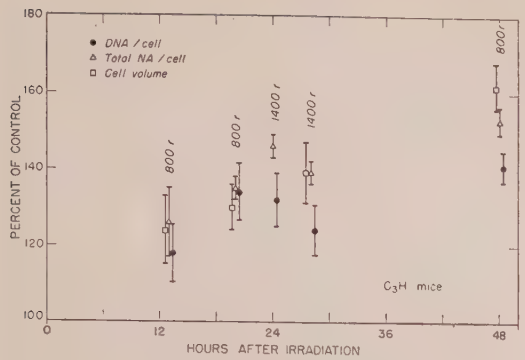

FIG. 2. Mean nucleic acid contents/cell* at varying times after radiation.

* All values expressed as % of controls. Vertical lines represent stand. errors of the ratios.

hour. At 4 days the control mitotic index was 1.2 ± 0.2%. After irradiation no mitotic figures were seen for the 2-day experimental period.

Fig. 1 represents DNA specific activities measured when P^{32} was injected at varying times after 800 r and the mice sacrificed 2 hours later. Each point is the mean of 5 determinations (10 mice) expressed as percentage of the control value measured simultaneously. As is evident from the standard error of the ratio there was considerable variability. However, the data indicate that the P^{32} incorporation into DNA was essentially normal for the first few hours after irradiation and above normal at 12 hours. Between 23 and 36 hours after irradiation the DNA specific activity was depressed to about half the control value. No significant alteration in the specific activity of cellular inorganic phosphate or organic acid soluble phosphate was observed throughout this time.

If the normal rate of incorporation of P^{32} into DNA after irradiation represented synthesis of DNA, there should be an increase in the total amount of DNA in the tumor. However, since there was no cell division and no increase in cell number during this time, the amount of DNA per cell should increase. Fig. 2 presents results of biochemical determinations of the mean DNA content per cell at several time intervals after 800 r. It also includes the mean total nucleic acid content per cell and mean cell volume. All values are expressed as percentage of controls measured simultaneously. A clear increase in all 3 quantities was observed after irradiation. The increase in mean cell volume and mean nucleic acid content per cell agree with the observations of Klein and Forssberg(2). Since the pronounced increase in the mean amount of DNA per cell did not agree with their findings (when they used 1200 r), 2 further experiments were performed with 1400 r which are included in Fig. 2.

The rate of increase in mean DNA content per cell during the first day post irradiation was 1.5% per hour. This is in agreement with the rate of increase in cell number in the unirradiated tumor during this time interval. It also compares well with the estimated rate of DNA formation of 1.7% from incorporation data in unirradiated cells. These data support the conclusion that incorporation of P^{32} into DNA of irradiated Ehrlich ascites cells is due to continuation of DNA synthesis during the first day post irradiation.

During the second day after irradiation the incorporation of P^{32} was markedly depressed (Fig. 1), and there was little further increase in mean DNA content per cell. To determine whether, during this time interval, a discrepancy existed between incorporation data and net synthesis of DNA, many more experiments would have to be carried out. In particular it would have to be shown that not even a small percentage of cells underwent mitosis or died.

Fig. 3 is a histogram of the distribution of individual DNA values (in arbitrary Feulgen units) in nuclei of control and irradiated ascites cells. It demonstrates a definite increase, after irradiation, in the proportion of cells with a high DNA content, confirming the biochemical findings. Whereas in the control tumor 32% of the cells had a DNA content above 11 (arbitrary units), 60% of the irradiated cells were above this value. It would be of interest to know whether the cells which still had the lower DNA content would have synthesized more DNA if given sufficient time, or if they represented cells damaged by irradiation or by the normal course of the tumor's development(14).

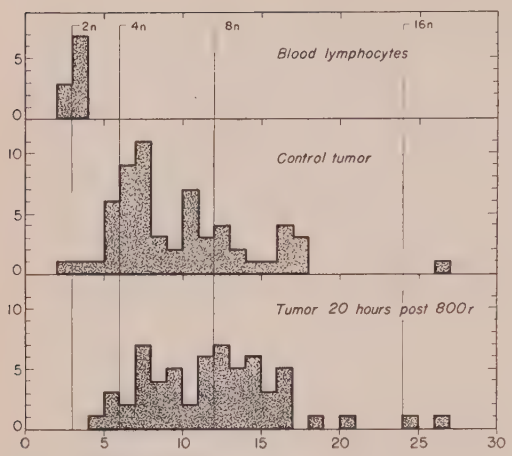

FIG. 3. Distribution of individual DNA values* in Ehrlich ascites cells.

* Abscissa: DNA content in arbitrary Feulgen units. Ordinate: No. of cells with given DNA content. Using 2n (diploid) value for DNA content of blood lymphocytes as reference point, other n values calculated in arbitrary Feulgen units.

The present experiment confirms once more the fact that mitosis may be inhibited after irradiation without simultaneous inhibition of DNA synthesis. The results would be consistent with the idea that although the cells are unable to go through mitosis, they continue to synthesize DNA until the majority of cells have reached the premitotic DNA content at which time the rate of DNA synthesis decreases. The Feulgen data, however, serve to emphasize the complexity of the cell population and the need for caution in interpretation of changes in mean values measured biochemically. The recent results of Harrington and Lavik(15) further suggest that there may be indirect factors involved.

Summary. Experiments were carried out 4 to 6 days after inoculation of approximately 2 x 10^7 Ehrlich ascites tumor cells in mice. When the 2-hour incorporation of P^{32} into DNA was measured at various times after irradiation (800 r total body x-irradiation), no significant depression in DNA specific activity was observed until 1 day. Measurements of mean cell volume, mean DNA content per cell, and mean total nucleic acid content per cell at 13 and 20 hours revealed that all 3 quantities increased at approximately the same rate, closely matching the growth rate of the unirradiated tumor and rate of DNA formation estimated from the incorporation data. The increased DNA content per nucleus after radiation was confirmed by Feulgen microspectrophotometry. At 48 hours after irradiation cell volume and total nucleic acid per cell had risen even higher while DNA per cell showed little further increase. The data suggest that the irradiated cells continue to synthesize DNA until they reach the premitotic DNA content (octoploid in Ehrlich cells) and are arrested there because they are unable to go through mitosis.

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Hypoxia at Normal Atmospheric Pressure As a Cause of Congenital Malformations in Mice.* (22862)

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In view of the finding that pregnant mice (1,2,3), rabbits(4) and rats(5) may be made to produce malformed progeny following exposures to low atmospheric pressures of only a few hours duration, and because the interpretation was advanced that these congenital defects were late manifestations of hypoxia the following study was undertaken to test the validity of this assumption. If the results were indeed due to hypoxia then correspondingly low concentrations of oxygen molecules at normal atmospheric pressure should be able to produce similar effects. In the following experiment, therefore, pregnant mice were made hypoxic at normal atmospheric pressure; and this report describes what effect that treatment had on the unborn progeny.

Methods and materials. The 22 white mice used were in their 10th day of pregnancy, a critical time for development of normal or abnormal embryonic ribs and vertebrae(2). Twelve of these mice were kept one or two at

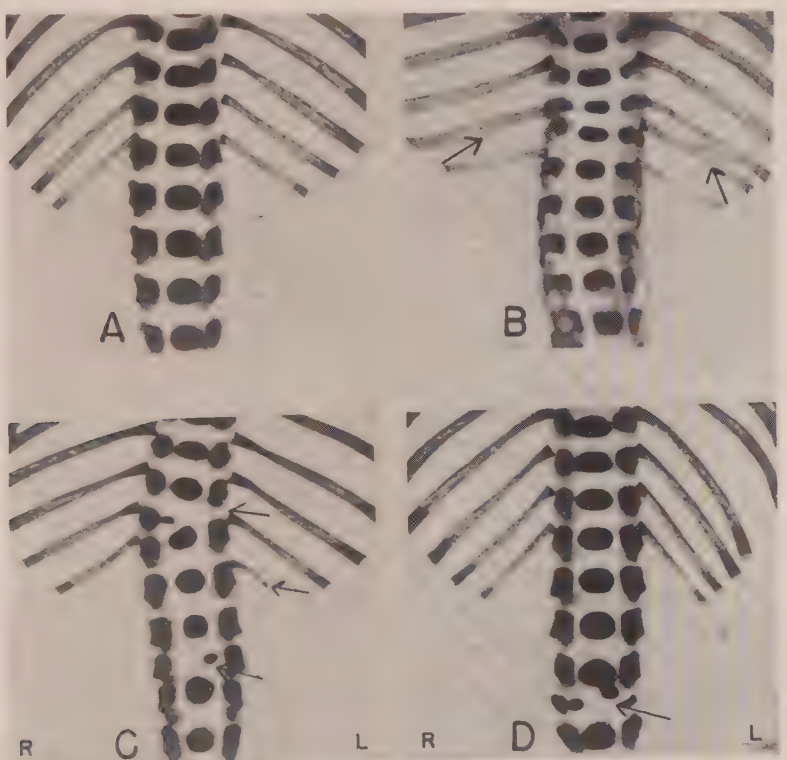

FIG. 1. Malformed vertebrae and ribs in mice on 19th day of gestation, after hypoxia on 10th day. A. Normal vertebrae and ribs—10th thoracic to 4th lumbar segments. B. Total fusion of ribs 11 and 12 on right side of thorax; partial fusion of opposite ribs. C. Ablation of 12th rib and vertebral transverse process on left—hypoplastic, extra 14th rib on same side; hemivertebra of 12th thoracic and 3rd lumbar segments. D. Hemivertebra and fusion of 3rd and 4th lumbar centrums.

* Aided by grants from Assn. for Aid of Crippled Children, United Cerebral Palsy; Amer. Heart Assn. and Mass. Lions Eye Research Fund.

a time for 2 hours in a bell jar of 3.5 liters capacity through which was flowed from bottom upwards a mixture of dry nitrogen and air containing 6% oxygen. A dental anesthesia machine was used to mix the gases and provide constancy of flow which was at a rate estimated at more than 1 liter per minute. A Hay's gas analyzer having graduated measurements of 0.2% was used to measure oxygen and carbon dioxide percentages at inlet and exhaust orifices of the bell jar. Carbon dioxide concentration never reached a measurable level and oxygen concentration remained at six percent; humidity, however, was not standardized in this apparatus. A nitrogen-oxygen mixture at sea level (atmospheric pressure, 760 mm mercury) containing 6% oxygen gives approximately the same concentration of oxygen molecules as exists in the atmosphere at 30,000 feet above sea level (atmospheric pressure, 226 mm mercury). As with mice exposed to low atmospheric pressure(2), test mice upon return to room air showed no immediate ill effects from the exposure and appeared well throughout pregnancy.

The 10 remaining mice were kept as controls under usual laboratory conditions. Fetuses were removed from mothers by section during the 19th day of gestation, and skeletal systems were stained with alizarin red for examination after clearing of soft tissues with potassium hydroxide.

Results. Exposure of pregnant female mice on 10th day of pregnancy for 2 hours to a 6% oxygen-94% nitrogen mixture at normal atmospheric pressure (760 mm of mercury) resulted in malformations of the kind found after exposure to a low atmospheric pressure—226 mm of mercury, which in respect to number of oxygen molecules per unit volume of air is the equivalent of 6% oxygen in nitrogen at sea level. Hemivertebrae, ablations and fusions of ribs and vertebrae (Fig. 1) were found in 26% (15 of 57 young) of the progeny of 12 females. None of 52 young from control animals kept in ordinary atmosphere at sea level showed similar defects.

Summary. Pregnant mice kept for 2 hours during the 10th day of gestation in a gas chamber containing 6% oxygen in nitrogen at normal atmospheric pressure produced progeny having malformed ribs and vertebrae.

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Transcapillary Exchange of S^{35}-Labeled l-Methionine in the Human Forearm.* (22863)

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Previous reports from this laboratory have been concerned with the transcapillary exchange of water and electrolytes in different vascular areas of the body(1,2). A technic was described utilizing the forearm vascular bed where the transcapillary exchange of labeled substances could be studied in some detail. Observations could be extended over a

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† Helen H. Millenson Memorial Fellow, Metropolitan Heart Guild, Washington, D. C.

TABLE I.

Patient	Age	Diagnosis	% loss around peak of dye curve		% loss of methionine	
			SCN	Methionine	30 sec. past peak	At equilibrium time of SCN
C.C.	32	Peptic ulcer	45	51	55	44
B.N.	50	Conv. pneumonia	30	11	33	40
G.K.	28	Psychoneurosis	47	41	54	45
G.S.	35	Irr. colon	44	36	39	34
W.A.	29	Pyelonephritis	49	11	42	28
P.N.	44	Peptic ulcer	41	14	49	49
B.B.	26	Psychoneurosis	40	18	49	24
E.B.	57	Gastroenteritis		56	49	
J.D.	71	Bronch. ca.		28	52	
J.W.	48	Essen. hypert.		19	54	
		Mean and S.D.	42.3 ± 6.3	28.5 ± 16.7	47.6 ± 7.3	37.7 ± 9.3

relatively long period of time since contamination by significant recirculation of the labeled materials could be avoided(3). It was found that water and thiocyanate ion crossed the capillary membrane from blood to tissue at net rates characteristic for each substance and that return of these substances from tissues to blood occurred relatively rapidly(3).

It seemed of interest to extend these studies to include an organic substance which is utilized in cellular metabolism in order to determine whether the pattern of transcapillary exchange of such a substance might differ from that observed previously with thiocyanate ion and isotopic water.

Methods. The present studies were performed on 10 male patients with various disorders as listed in Table I. Their average age was 42 with a range from 26 to 71 years. The method of administration and collection of samples, which has been described previously (1), consists of injecting into the brachial artery a mixture of the substance under study, followed by continuous sampling at intervals of 2 to 4 seconds from an ipsilateral antecubital vein. The dye T-1824, and sodium thiocyanate were given in the same amounts as described previously(1). To this mixture S^{35}-labeled l-methionine was added to provide an injected activity of 25 to 30 μc. Periodic samples from a contralateral vein revealed that the concentration of recirculating tracer materials was insignificant. Analytical procedures for determining concentrations of T-1824 and thiocyanate have been described(1). Aliquots of plasma samples were diluted with equal parts of isotonic saline solution and 0.5 ml of each was then pipetted into aluminum planchets in order to count S^{35}-beta emissions. Aliquots of the injected mixture first were diluted 200 times with isotonic saline solution, mixed, and an aliquot of this was diluted with equal parts of plasma containing no radioactivity. 0.5 ml of the latter was also transferred to a planchet. Samples were dried at room temperature and counted, using a thin, end-window ("Micromil") gas flow counter.‡ The method for calculating the net transcapillary losses of thiocyanate has been described(1,3). The same method was applied to S^{35}-labeled l-methionine. Correction for red cell penetration of methionine was determined *in vitro* by comparing the activity of S^{35}-labeled l-methionine added to red cell free plasma with that added to whole blood. The amount added was .002 μc/ml plasma or whole blood. Whole blood samples were allowed to equilibrate one hour, then centrifuged, and the activity of the supernatant plasma and also of the reference plasma samples determined as described above. Counts/min/ml in supernatant plasma multiplied by the plasmatocrit gave total counts in the plasma fraction of 1 ml whole blood (C_{pwb}). Red cell penetration (C_{rc}) was the difference between C_{pwb} and the counts/min in 1 ml of reference (red

‡ Model No. D-47, Nuclear Instrument Corp., Chicago, Ill.

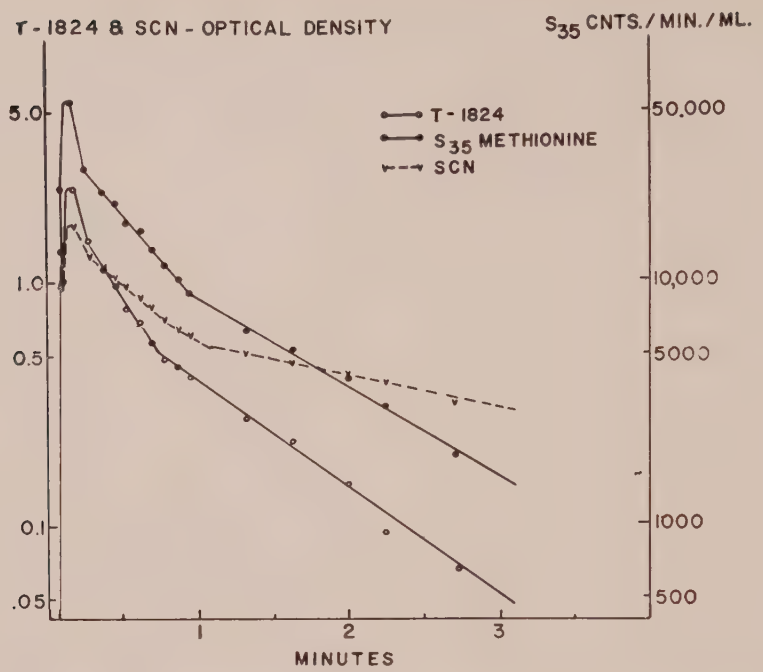

FIG. 1. Time concentration curves obtained from antecubital vein after inj. of a mixture of the dye T-1824, sodium thiocyanate and S^{35}-labeled l-methionine into the ipsilateral brachial artery in subject G.S. The T-1824 and methionine downslopes are nearly parallel. The thiocyanate curve deviates from the others soon after the peak. See text for further details.

cell free) plasma. The mean value for C_{rc}/C_{pwb} in blood samples drawn from 5 individuals was 0.54 (range .47 to .64). The penetration factor was expressed as the fraction of red cell water available for equilibration with plasma water and was obtained by multiplying the mean value by the ratio of the water content of plasma to the water content of red cells ($0.54 \times .90/.65 = .75$). This penetration factor (0.75) was multiplied by $\frac{cWc}{pWp}$ [see equation 4 of the original description of methods(1)]. In the case of SCN a penetration factor of 0.70 rather than 1.0 was used in calculating the expected concentration of the thiocyanate(2).

Results. I. *Characteristics of time-concentration curves.* The downslopes of time concentration curves of S^{35}-labeled l-methionine differed from those previously observed with deuterium oxide and thiocyanate ion(3). The methionine downslope paralleled the T-1824 downslope much more closely than thiocyanate ion (Fig. 1). This parallelism usually persisted beyond equilibrium time (3) of thiocyanate and only when the concentration of injected substances had reached almost trace levels did the methionine curve assume a distinctly shallower slope than the T-1824 curve. In 7 instances the early portion of the methionine downslope was somewhat steeper than that of T-1824.

Plotting "expected" and actual concentrations(3) of labeled methionine revealed a delayed equilibrium time and only insignificant return as compared to thiocyanate (Fig. 2). In contrast to SCN and D_2O, a half return time(3) could not be calculated for methionine because of the late onset and small quantity of return during the period of observation.

II. *Transcapillary Loss.* In the case of thiocyanate and deuterium oxide, the greatest transcapillary loss occurred near the peak of the concentration curves at a time when the concentrations in blood exceeded that in extravascular spaces(3). In the case of labeled methionine the per cent loss at the peak was somewhat variable and usually rose to a higher level during the half minute follow-

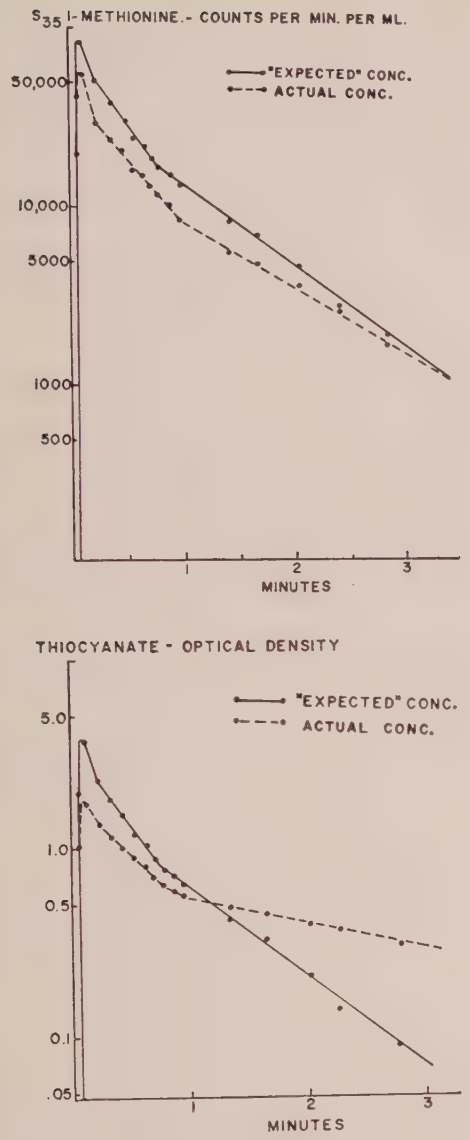

FIG. 2. "Expected" and actual concentration curves of S^{35}-labeled l-methionine (above) and of thiocyanate (below) derived from Fig. 1. See text and reference 3 for further details. This case did not show the increasing % loss in the portion immediately following the peak as did most of the other cases.

ing the peak where it remained fairly constant for a considerable period. Thus, the mean per cent transcapillary loss of labeled methionine at the peak in the 10 cases studied was 28.5 with a large standard deviation of 16.7. The per cent loss at a point 30 seconds beyond the peak concentrations averaged 47.6 ± 7.3. The small percentage return of these transcapillary losses is indicated in the 6 cases in which SCN was determined simultaneously. At the equilibrium time of thiocyanate [point at which net return equals net loss(3)] the net exchange of labeled methionine averaged 37.7 ± 9.3% loss from blood to extravascular spaces.

The per cent loss of thiocyanate ion observed in these studies was in the same range as that previously reported(3). In the 6 cases studied in the present experiments the mean transcapillary loss at the peak was 42.3 ± 6.3%.

Discussion. The observed transcapillary losses of labeled methionine indicated that this substance diffused relatively freely through the capillary walls. Despite this free diffusion, however, the net return from extravascular space to intravascular space was minimal during the period of observation. These facts suggest that the labeled methionine became bound after leaving the circulation in such a way as to prevent its return. This would explain the persistence of maximal rates of net transcapillary loss despite a constantly diminishing concentration in the blood. The most reasonable site of such binding would be metabolic incorporation into cells. Cowie and Roberts have presented evidence for a high degree of metabolic binding of S^{35}-labeled amino acids in bacterial cells (4). Intracellular penetration and binding of l-methionine would explain the difference in the pattern of transcapillary net exchange as compared to the largely extracellular thiocyanate ion. It is significant that other small molecules which have primarily an extracellular distribution, such as S^{35}-labeled sulfate and radiosodium exhibit transcapillary exchange patterns resembling thiocyanate ion (5).

Still a third pattern of transcapillary net exchange was exhibited by heavy water which showed early transcapillary losses averaging 90%, a delayed peak compared to T-1824 and equilibrium and half-return times intermediate between SCN and methionine(3). These differences between the diffusion patterns of D_2O and methionine can be ac-

counted for on the basis of the following characteristics of D_2O: (1) extracellular as well as intracellular distribution, (2) unusually high diffusibility through capillary walls, and (3) escape of a considerable portion from metabolic binding.

The mechanism for the frequently observed increasing per cent net transport during the early period of the downslope of l-methionine is not apparent. Possible explanations might be that metabolic incorporation is not as efficient when peak concentrations are present, or that the later downslope represents plasma moving at lesser velocity permitting more complete transcapillary exchange.

Summary and conclusions. 1) In the forearm the maximum transcapillary loss of S^{35}-labeled l-methionine averaged 47.6 $\pm$ 7.3%. Methionine exhibited a pattern of transcapillary net exchange which is consistent with intracellular binding. A high rate of net loss was observed for a much longer period than with thiocyanate ion and the equilibrium time was delayed considerably. Net return to the circulation was small compared to thiocyanate during the period of observation. 2) On the basis of these and other observations it is suggested that 3 distinct classes of substances have characteristic patterns of transcapillary net exchange which may be distinguished with the present technic. These substances are (a) primarily extracellular ions, (b) primarily intracellular diffusible substances, and (c) highly diffusible molecules which are distributed throughout both compartments (body water).

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Changes in Plasma Prothrombin, Ac-Globulin, and Antithrombin Concentration Following Intravenous Administration of Estrogens.* (22864)

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Recent observations made in clinics indicate that estrogens given intravenously are of therapeutic value for control of spontaneous hemorrhage, such as epistaxis, excessive postoperative bleeding from tonsillectomies and, on occasion, from urological surgical procedures(1,2). Moderate success has also been reported for this therapy in spontaneous gastrointestinal bleeding. These uses are of more recent application than for control of uterine bleeding for which estrogens have had long popularity. The studies here reported are regarded as a preliminary survey designed to determine what changes may be produced in concentrations of blood coagulation factors following intravenous injection of estrogens. Previous investigators reported alterations of clotting factors coincident with physiological changes in levels of estrogenic hormones, as well as a rise in heparin like antithrombinic activity with administration of synthetic estrogen(3). For a brief period ovarian extracts were given extensive trials for control of bleeding tendency in hemophilia(4), but this did not become established practice(5). During pregnancy there is elevation of prothrombin level(13) near term as well as in

* Aided by a grant from Ayerst and Co., New York City.

serum prothrombin conversion accelerator (6) and fibrinogen(7). During the menstrual cycle, platelet numbers increase at ovulation and fall at menses(8). With administration of 20 mg of estrogens intravenously prompt cessation of hemorrhage has been noted(2). Occasionally bleeding commenced again after 4 to 5 hours but with repetition, bleeding stopped. Toxic effects or side reactions have not been noted for these short term administrations although prolonged use might lead to endocrinological disturbances.

Material and methods. The estrogenic preparation, Premarin, is described as amorphous complex containing sulphates of steroids occurring in pregnant mares urine. The preparation also contains small amounts of nonsteroid phenol sulphates. The preparation of laboratory reagents for investigation of blood clotting mechanisms has been described previously as follows: Purified prothrombin(9), purified thrombin(10), bovine platelet suspension(11), assays of prothrombin(12), thrombin(14), fibrinogen(15), Ac-globulin(16), antithrombin(17), autoprothrombin I(18), platelet cofactor I(19), and platelet cofactor II(20).

Results. Prior to intravenous injection of Premarin, it was used in the test tube as an added or substituted factor in analyses mentioned above without significant change being noted in any of the assays.

Dogs of both sexes were anesthetized with Nembutal. A control blood specimen was drawn using 3.2% sodium citrate as anticoagulant. Ten mg of estrogen preparation was then given intravenously and blood specimens were drawn at intervals using a 2-syringe technic to avoid contamination of specimens with tissue thromboplastin. Plasma was obtained by centrifugation. A sample of blood was also drawn to obtain serum. Usually analyses were then done at once or the specimens were frozen and stored in deep freeze for analysis the next day. Larger doses of estrogens were given to 2 dogs and there was a proportionately greater change in measured values in the same direction as with the small dose. Nine male and 3 female dogs were studied. All indications from these animals confirm the selected example recorded in Table I.

TABLE I. Changes Observed in Coagulation Factors after Intravenous Injection of Estrogens.

Time, min.	Prothrombin, units/ml	Ac-globulin, units/ml	Antithrombin*
Control	134	32	790
	(Premarin inj. of 10 mg given intrav.)		
15	162	36	960
30	185	90	880
55	190	70	860
130	152	86	890
150	175	100	760
240	145	72	700

* Expressed as units of thrombin remaining after incubation for 2 hr with a substrate of about 1400 units of thrombin/ml.

Discussion. Elevation of prothrombin values as well as depression of antithrombin content of plasma, increases the amount of potential thrombin available and also tends to make it more effective. This allows thrombin to manifest its multiple functions in the clotting process to a greater degree, particularly in the presence of increased amounts of Ac-globulin.

The values for Ac-globulin rose markedly. In the dog, these values are normally high; in the human, the normal values are only 10 to 15 units/ml. The latter figure is one of the lowest of all species tested(21), so that depression of a few units may be of importance in the etiology of hemorrhage. Conversely, an elevation might be of importance for the prevention or control of bleeding. Should human plasma have a rise in Ac-globulin values following estrogen administration proportional to that in the dog, this would be of unusual advantage to man.

Summary. Solutions of estrogenic steroids were administered intravenously to dogs. There was a sharp rise in the plasma Ac-globulin and prothrombin concentration. These changes began within the first 15 minutes, reached a peak in about 1 and ½ hours. In about 3 or 4 hours normal concentrations were again found. A small decrease in antithrombin activity of plasma was found. Theoretically all these changes tend to enhance coagulability of the blood.

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Increase in Tolerated Dose of Isoniazid in Mice by Use of Cycloserine. (22865)

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Isoniazid continues to be in widespread use in the therapy of human tuberculosis. The toxicity of the drug is a limiting factor in dosage. In animal experiments with this compound, it has been found possible to increase the tolerated dose by the use of suitable detoxifying agents(1). Such agents were also employed to investigate the possible detoxication of the new antibiotic cycloserine,* D-4-amino-3-isoxazolidone, of current interest in the treatment of human tuberculosis(2-4). Unexpectedly, it was observed that simultaneous administration of isoniazid (INH) and cycloserine (CS) resulted in a slight reduction of the hyperactivity induced in mice by the CS, and marked reduction in the percentage mortality in mice given larger, otherwise lethal, doses of INH. This latter observation prompted the present detoxication study with these two chemicals.

Methods. Control toxicity tests of each chemical by gavage in DBA mice showed that (a) INH permitted 45% survival with a 5 mg dose and no survival with 6 mg (250 and 300 mg/kg respectively)(1); and (b) CS was tolerated by 6 of 7 mice each given 250 mg, but was 100% lethal with 300 mg (12.5 and 15 g/kg respectively). CS in amounts of 25 mg or more caused hyperactivity evidenced by continuous circling in the jar beginning about 30 minutes after administration of the chemical.

Results. Tolerance limits after a single dose of mixed chemicals. The effect of a single oral dose of mixtures of various concentrations of CS and INH on survival time of

* Kindly supplied as seromycin by Eli Lilly and Co., Indianapolis, Ind.

TABLE I. Effects of a Single Administration of Mixtures of Isoniazid and Cycloserine at Designated Concentrations in DBA Mice.

Isoniazid, mg	Cycloserine, mg	No. of mice	Survival of mice	
			No.	%
0	100	25	25	100
0	250	7	6	86
0	300	4	0	0
4	0	10	10	100
5	0	49	22	45
6	0	10	0	0
8	25	18	8	44
8	50	18	13	72
8	75	15	15	100
10	50	14	11	79
10	75	16	13	81
10	100	22	22	100
15	100	11	7	63
15	200	11	4	36

DBA mice is reported in Table I. The 2 chemicals in appropriate amounts were mixed in a test tube and dissolved in water to such volume that the desired dose of each was contained in 0.5 ml volume. A single administration of 8 mg INH plus 75 mg CS, or of 10 mg INH plus 100 mg CS, per 20-gram mouse permitted 100% survival. As much as 15 mg INH with 100 mg CS were tolerated by 7 of 11 mice (63%), but with larger amounts of CS the mixtures were toxic.

Survival time after repeated doses of mixed chemicals. Repeated administration of a mixture of 10 mg INH with 100 mg CS was attempted on successive days; however, after the second dose, 5 of 6 mice succumbed. These same concentrations were then administered twice weekly (Mondays and Thursdays) for 10 weeks. All of the 6 mice tolerated 14 doses and 5 withstood 20 doses with no loss in weight but a normal gain over the period. This observation was confirmed in 2 similar experiments. As seen in Table II, the composite of the 3 experiments yielded a 73% survival after 20 doses. In one control experiment, 6 animals given CS only tolerated 17 doses; one died after the 18th dose, and all appeared "sick" after the 19th and 20th doses when the experiment was terminated. A second control group of 10 mice reached a 50% endpoint with 11 doses; the survivors, given 2 more doses, were observed

TABLE II. Survival Time after Repeated Doses of Mixed Chemicals, Isoniazid and Cycloserine, in DBA Mice. Administration twice weekly for 10 weeks.

Isoniazid, mg	Cycloserine, mg	No. of mice	No. of mice surviving following No. of doses			
			1	7	13	20
0	100	6	6	6	6	5
0	100	10	10	8	5*	
10	100	26†	23	23	22	19

* Discontinued after 13 doses; subsequent deaths occurred on 7th, 9th, 14th, and 21st day.
† Composite of 3 exp.

for 21 days. Four of the 5 mice died in that interval. It is significant that the hyperactivity due to CS, although not as marked as with this drug alone, was noticeable after each administration of the mixed drugs. Thus it would appear that CS permitted the mice to tolerate almost double the amount of INH which was lethal *per se*.

Blood plasma concentrations. At the end of the above long-term experiment, studies were made of blood plasma concentrations of the 2 chemicals. INH content was measured by the "pyridyl" method(5), and CS by the Jones method(6). It had been previously determined that there was no interference in these 2 methods. The plasma 72 hours after the 20th administration contained 0.38 mg % of INH and no detectable amount of CS. The CS control blood plasma level, 24 hours after the 20th dose, was 0.68 mg %. This figure is to be contrasted with the 24-hour level following a single injection: for from an absorption curve with this compound, it was found that 100 mg CS alone gave the following levels: at 2 hours, 11.2 mg %; at 4 hours, 8.7 mg %; at 7 hours, 5.0 mg %; and at 24 hours 0.1 mg %. Thus apparently repeated doses of CS alone resulted in a slight cumulative effect which might account for the "sick" appearance of the animals in the last week of the 70-day experiment. This was not observed in the mice given the mixture of INH with CS.

Effect on in vitro *Inhibition Tests.* One method has been employed for testing whether these proportionate amounts of CS and INH have any effect on the *in vitro* inhibitory activity of either compound against *Mycobacterium tuberculosis.* Following the

procedure described earlier(1), it was found in replicate experiments that as much as 200 μg per ml of CS alone had no inhibitory action on the growth of strain B103; INH alone inhibited in a dose of 5 to 10 μg per ml; and with the mixture, concentrations in the proportion of 100 μg CS and 10 μg INH per ml gave an inhibition endpoint the same as with INH alone. Similar experiments with 24-day growth of 3 other strains, 198ARB, H37Rv, and BCG, although demonstrating variations in response to CS alone (inhibition with 200, 20, 10 μg, respectively) were similar in their inhibition results with INH (5 to 10 μg) and with the mixture (10 μg INH). One may conclude from these *in vitro* tests that at least the presence of CS under the conditions used did not interfere with the inhibitory action of INH.

Summary. The acute toxicity of isoniazid (INH) in DBA mice by gavage, $LD_{50} = 5$ mg/20 g mouse, may be reduced by simultaneous administration of the antibiotic cycloserine (CS). Each of 22 mice survived a single dose of 8 mg INH with 75 mg CS (0.4 g and 3.75 g/kg respectively), or of 10 mg INH with 100 mg CS (0.5 g and 5.0 g/kg respectively). On repeated semi-weekly administration for 10 weeks, 19 of 26 mice, 73%, survived the latter mixture. Significant amounts of INH were detected in the blood plasma 72 hours after the 20th dose. In *in vitro* bacteriostatic tests with 4 strains of *Mycobacterium tuberculosis,* CS did not interfere with the activity of the INH.

Since submission of this manuscript for publication, a statement in a technical bulletin indicated that in clinical use "toxicity (of CS) is decreased and its efficacy increased" when administered with INH. (*Phys. Bull.,* 1956, v21, 227).

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Localization of Lactic Acid Dehydrogenase Activity in Serum Fractions. (22866)

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The enzyme lactic acid dehydrogenase (LDH) is known to catalyze the conversion of pyruvic acid to lactic acid in the presence of reduced diphosphopyridine nucleotide (DPNH)(1,2). More recently Vallee has shown that LDH is a zinc protein(3). Increased interest in the level of LDH in various disease states has followed reports that in patients with myocardial infarction and leukemia the serum level of LDH is increased significantly(4,5,6,7).

The main purpose of this paper is to describe 3 separate peaks of LDH activity in the electrophoretically separated fractions of the serum proteins.

Methods and materials. Twelve subjects, 3 with leukemia, 2 with myocardial infarction, 2 normal individuals and 5 patients with such various diseases as cirrhosis of liver, hemochromatosis and Wilson's disease, were employed. Three to 5 ml of fresh, non-hemolyzed serum were separated by zone electrophoresis using a starch or polyvinyl supporting medium in barbital buffer pH 8.6 ($\pi/_2$

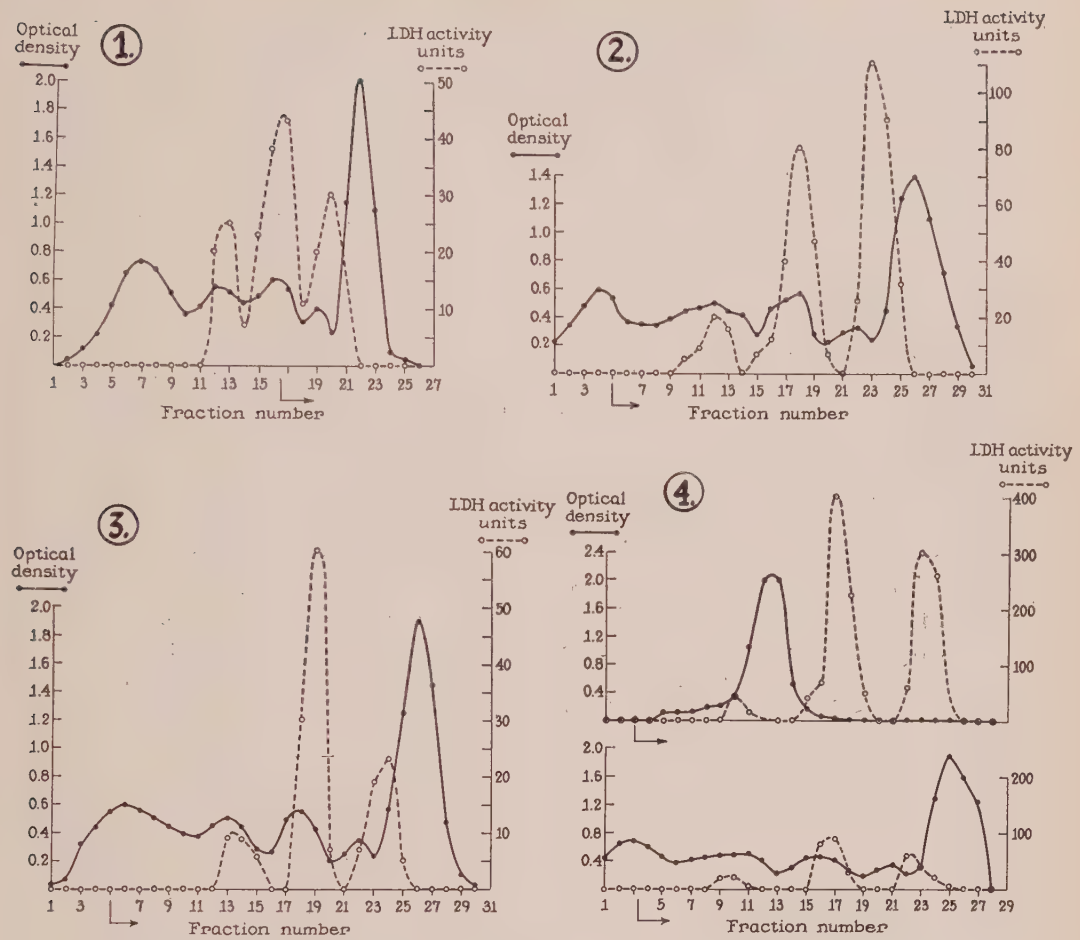

FIG. 1. Curves showing distribution of lactic acid dehydrogenase in normal human serum.

FIG. 2. Distribution of lactic acid dehydrogenase in serum of patient with myocardial infarction.

FIG. 3. Distribution of lactic acid dehydrogenase in patient suffering from myelogenous leukemia.

FIG. 4. Upper curve represents distribution of lactic acid dehydrogenase in hemolysate from human red cells. Lower curve represents the localization of lactic acid dehydrogenase of the serum from the same patient separated under identical conditions on the same starch block.

$= 0.1$). After electrophoresis the starch or polyvinyl block was cut into half-inch segments, protein was eluted and its concentration determined by modification of Folin-Cioculteau procedure(8). An aliquot of each eluate was then analyzed for LDH activity by the method of Wroblewski and LaDue(4) which depends on oxidation of the coenzyme DPNH to DPN according to the equation

$$\text{Pyruvate} + \text{DPNH} \underset{\text{Lactic acid dehydrogenase}}{\rightleftharpoons} \text{Lactate} + \text{DPN}$$

Sixty to 90% of the LDH activity of the original serum was recovered after electrophoresis and elution. To obtain maximum activity of the fractions complete recovery was not always attempted. However, if larger volumes of eluate were used, recoveries up to 90% could be obtained. A water flow experiment showed that all the LDH migrated with the protein band, none being differentially absorbed by starch. In several experiments rabbit serum and hemolysate from human red cells were separated electrophoretically and assayed for LDH activity.

Results. Each of the 12 sera separated

TABLE I. LDH Activity in Serum Fractions.

	No. of cases	Total activity units	% activity in serum fraction β	α_2	α_1	α_2/α_1
Controls	6	284 (193–370)	19 (12–23)	47 (43–55)	34 (31–37)	1.5 (1.3–1.6)
Myocardial infarction	2	626	10	38	52	0.7
		1892*	4	35	61	0.6
Leukemia	3	500	18	57	25	2.4
		180	13	57	30	1.9
		198	17	55	28	2.0

* Extensive infarction (fatal).

electrophoretically and assayed for LDH activity revealed 3 distinct loci of activity (Fig. 1). One peak of activity was found in the β-globulin; a second peak of activity was found in the α_2-globulin; and the third occurred between the α_1-globulin and the albumin, hereafter referred to as the α_1 peak. Addition of excess ionic zinc to the serum prior to electrophoresis did not alter the pattern of LDH activity obtained. Since it is known that red cells are particularly rich in the enzyme LDH special care was taken to avoid excessive hemolysis. Presence of trace hemolysis, however, was not found to increase the serum LDH activity significantly. Dialysis of the serum prior to electrophoretic separation likewise did not alter the results obtained.

In the 6 control subjects each peak of LDH contained a relatively constant percentage of the total LDH activity found in the serum. Nineteen % of total LDH activity was found in the β-globulin, 48% in the α_2-globulin and 33% in the α_1 peak. The variability of the percentages found in the 3 fractions on the control subjects is shown in Table I. The greatest variation appeared in the β peak. In control subjects the ratio between the LDH activity in the α_2 peak and the α_1 peak was approximately 1.5.

In sera obtained from 2 cases of myocardial infarction in which total LDH activity was raised the α_1 activity peak was increased becoming larger than the α_2 peak (Fig. 2), and thus the α_2/α_1 activity ratio decreased. Three cases of leukemia, 2 myelogenous and one lymphatic were studied. In the patient with lymphatic leukemia and the patient with myelogenous leukemia in whom the white count was not raised, no significant increase in serum LDH was observed. However, one patient with myelogenous leukemia, in whom the white count at time of examination was 33,000, showed an increased serum LDH activity. Despite the fact that only one of the 3 cases showed an absolute rise in LDH activity all showed a significant increase in the α_2 activity peak relative to the α_1 peak and thus the α_2/α_1 ratio became greater (Table I).

When a hemolysate from human red cells was separated electrophoretically 3 peaks of LDH activity were identified. These 3 activity peaks approximately corresponded in their electrophoretic mobility and their relative concentrations to those found in serum. From the point of view of the isolation and purification of LDH it is of interest that the LDH activity protein ratio was greatly increased in the hemolysate compared to that found in serum. In the red cell hemolysate both the α_1 and α_2 activity peaks occurred in regions where the protein concentration was extremely low (Fig. 4). Considerably greater LDH activity was found in the hemolysate than in the serum.

In contrast to human serum, rabbit serum separated electrophoretically revealed two principal peaks of LDH activity. One of these occurred in the α_2-globulin region while the second and larger peak appeared in the α_1-globulin region.

In view of the finding that LDH is a zinc enzyme a study of the localization of radioactive zinc in the various serum proteins was carried out. Radioactive zinc was added to human and rabbit serum *in vitro* and electrophoresis performed. In both species 2 peaks of radioactivity were observed. In man the larger peak occurred in the slow moving part of the albumin (in some experiments coin-

ciding with the albumin peak) and the smaller peak migrated in the a_2-globulin region, in a position similar to the a_2 peak of LDH. Details of these experiments will form the substance of a separate communication.

Discussion. Since previous investigators have concluded that measurement of LDH activity in serum, based on change in optical density of the coenzyme is specific (2,4,5,6,7) the finding of 3 distinctly defined regions of LDH activity in serum fractions was a somewhat unexpected observation.

The rise in serum LDH reported in patients with myocardial infarction(4,5,7) was confirmed in the present study. However, it was of interest that the increase in LDH activity was restricted, in large part, to a rise in the a_1 peak with a consequent decrease in the a_2/a_1 ratio. In the 3 cases of leukemia although the total LDH activity of serum was raised in only one instance, thus confirming the inconstancy of an elevated serum LDH in leukemia(7), an increase in the a_2 peak relative to the a_1 peak occurred in all cases and the a_2/a_1 activity ratio was significantly raised. It thus appears that considerably more information can be obtained concerning LDH activity in disease states when fractionation is carried out than when the measurement of LDH activity is confined to whole serum.

The possibility that the 3 activity peaks might correspond to different forms of LDH elaborated from different sites is tentatively raised. It should be emphasized, however, that thus far the 3 peaks are demonstrably similar only in that they all exhibit LDH activity. More work on the detailed kinetics of the enzymatic reaction in the 3 peaks is required before it can be concluded that they do not represent different enzymes which react with a common substrate. It is of interest that Bajusz and Kovary(9) in investigating serum dehydrogenase by an entirely different method found 3 distinct spots on filter paper. In these experiments an indicator dye (2,3,5-triphenyltetrazolium chloride) was used. Unfortunately, details of the experiments are lacking.

The distribution of radioactive zinc in the serum fractions suggests that in both the rabbit and man, zinc is located principally in two electrophoretic regions. Since LDH contains zinc as an integral part of its molecule it was of interest that the a_2 radioactivity peak coincided approximately with the LDH peak in the a_2 region. However, further experiments are necessary to establish a definite relationship since the a_2 fraction is known to be a mixture of many proteins.

Summary. 1. Using the method of Wroblewski and LaDue the LDH activity in serum has been localized to 3 electrophoretic regions. In 6 control subjects approximately 48% of the LDH activity in serum was present in the a_2-globulin, 33% between the a_1-globulin and albumin and 19% in the β-globulin. 2. Three peaks corresponding approximately to those found in serum were observed in the hemolysate from human red cells. In the 2 cases of myocardial infarction in which the total LDH activity was increased the a_1 activity peak was differentially raised while in 3 cases of leukemia, although the total activity was increased in only one instance, the percentage of the total LDH activity in the a_2 peak was uniformly increased. 3. The results obtained suggest that an examination of the LDH activity in the different serum fractions may provide a more sensitive index of alterations of LDH activity than examination of the total serum concentration of the enzyme. 4. The distinguishing chemical and physical properties of the enzymes in the three peaks, in addition to the observed electrophoretic difference, remain to be studied.

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Effect of Different Dietary Fats on Choline Requirement of Rats.* (22867)

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Channon and Wilkinson(1) studied the effect of various dietary fats on level of liver fat in rats fed on low choline diets. They concluded that an increase in quantity of long chain saturated fatty acids in the diet caused increased liver fat deposition. Hartroft(2) has cited a number of observations which support the conclusion of Channon and Wilkinson(1). Work in this laboratory(3) with choline-free diets further substantiated this conclusion and also demonstrated that fats containing long chain saturated fatty acids caused increased liver fat deposition when fed with 9% casein diet containing 0.15% of choline chloride.

In none of these studies has it been determined whether the long chain saturated fatty acids increase the amount of choline needed to reduce the level of liver fat. Best, Lucas, Ridout, and Patterson(4) have pointed out the importance of using dose response curves in studying lipotropic factors. Therefore, a study has been made of liver fat deposition in rats fed on diets containing either butter fat or corn oil with various levels of choline.

Methods. Male rats of Sprague-Dawley strain weighing 70 to 84 g were used in Exp. 1. In Exp. 2 and 3, male rats of same strain weighing 40 to 50 g were used. The rats were separated into similar groups of 6 according to weight and were housed in individual cages with raised screen floors. During experimental period of 2 weeks the rats were fed *ad libitum*. The diet in first experiment contained in per cent: casein, 8; gelatin, 12; salts IV(5), 4; cellulose,† 2; sucrose, 44; and fat (as specified in the Table), 30. Vitamins were added to give as mg/100 g of diet: calcium pantothenate, 2; niacin, 2.5; riboflavin, 0.5; thiamine hydrochloride, 0.5; pyridoxine, 0.25; inositol, 10; biotin, 0.01; folic acid, 0.02; and vit. B_{12}, 0.002. Two drops of halibut liver oil fortified with vit. E and K (6) were given orally each week. Diets used in Exp. 2 and 3 contained 9% of casein instead of 8% and did not contain cellulose. All alterations in diets were compensated by adjusting level of sucrose. Butter fat was prepared by melting butter and decanting the fat layer through cheese cloth. At the end of the 2 weeks experimental period, the rats were stunned by blow on head and decapitated. Livers were removed and stored at —4°C until levels of liver fat were determined. Liver fat was determined by ether extraction of dried and ground liver(7). Those rats from which kidney sections were made, were killed in same manner. The left kidney was immediately removed and a portion of the organ was fixed with Bouin's Solution. Paraffin sections were stained with hematoxylin and eosin.

Results. Growth and liver fat results for the 3 experiments are presented in Table I. In the first experiment the basal diet was fed without amino acid supplements. Seven levels of choline were fed with corn oil and with butter fat. When no choline was added to diets, butter fat promoted a higher level of liver fat than corn oil. Choline reduced the level of liver fat with both dietary fats. Liver fat values were within the normal range when 0.12% of choline chloride was included in diets containing corn oil and when 0.15% of choline chloride was included in diets containing butter fat.

The groups receiving 0.04% of choline chloride or greater, grew at about the same rate whether corn oil or butter fat was fed. However, with no choline or only 0.02% of choline chloride, groups receiving corn oil grew at a slower rate, while groups receiving butter fat grew as well as those receiving higher levels of choline.

The basal diet is low in methionine, cystine, and tryptophan. Since dietary methionine and cystine and rate of growth are

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† Solka-Floc, Brown Co., Berlin, N. H.

TABLE I. Liver Fat and Growth of Rats Fed Corn Oil or Butter Fat with Levels of Choline.

Exp. No.	Supplement	Choline chloride, %	Butter fat		Corn oil	
			Liver fat, % dry liver*	Gain, g/wk*	Liver fat, % dry liver*	Gain, g/wk*
1	None	.00	67.4 ± 2.0	13.2 ± 2.5	51.0 ± 2.8	6.9 ± 2.6
		.02	58.1 ± 4.2	13.4 ± 2.2	42.0 ± 4.4	8.3 ± 3.2
		.04	50.5 ± 3.5	12.2 ± 2.5	28.7 ± 2.0	10.4 ± 3.0
		.08	31.2 ± 5.5	13.4 ± 2.0	21.7 ± 1.7	11.0 ± 2.2
		.12	20.8 ± 1.0	13.8 ± 2.5	16.3 ± 1.7	11.0 ± 1.0
		.15	16.6 ± .5	12.2 ± 3.8	17.7 ± .7	13.6 ± 2.0
		.30	14.0 ± .4	8.4 ± 2.6	15.7 ± 1.4	10.3 ± 2.2
2	None	.00	61.8 ± 1.7	11.6 ± 1.9	47.6 ± 2.7	6.2 ± 1.6
		.04	52.1 ± 3.1	13.8 ± 1.0	36.4 ± 2.2	14.2 ± 1.1
		.12	18.7 ± 1.0	17.3 ± 1.2	16.5 ± 1.4	14.8 ± 1.4
	Meth + trypt†	.00	62.7 ± .3	26.1 ± 1.3	51.9 ± 2.3	19.0 ± 2.6
		.04	46.8 ± 2.2	27.1 ± 1.3	31.3 ± 2.5	19.6 ± 1.7
		.12	29.8 ± 1.0	28.3 ± 1.8	18.2 ± 1.2	22.7 ± 1.7
		.15**	21.6 ± .6	21.1 ± .5	18.0 ± 1.1	21.3 ± .5
		.30**	22.1 ± 1.1	19.3 ± .4	15.9 ± 1.1	18.3 ± 1.6
	Cyst‡	.00	62.0 ± 2.9	8.9 ± 1.9‖	63.4 ± 4.0	10.9 ± 2.8¶
		.04	55.4 ± 4.5	18.5 ± 1.2	46.3 ± 4.0	15.0 ± 2.5
		.12	30.0 ± 3.8	13.1 ± .9	11.9 ± 1.3	15.3 ± 1.6
		.15**	17.4 ± 1.1	9.5 ± .8	13.7 ± 1.3	9.8 ± .7
		.30**	15.8 ± .4	11.0 ± .9	12.4 ± 1.0	9.9 ± .9
3	Meth + trypt + cyst§	.00	62.3 ± 1.3	19.8 ± 1.1	51.5 ± 1.5	15.2 ± 1.2
		.04	38.1 ± 5.7	18.9 ± 1.0	30.5 ± 2.7	19.9 ± 2.1
		.12	28.0 ± 1.9	19.2 ± .8	16.6 ± 1.6	17.2 ± 1.4
		.30	21.5 ± 1.4	17.7 ± 1.1	16.0 ± .8	19.5 ± 1.6

* The mean ± stand. error of mean for 6 rats. † .3% DL-methionine + .1% DL-tryptophan. ‡ .3% L-cystine. § .3% DL-methionine + .1% DL-tryptophan + .3% L-cystine. ‖ Two rats died before end of exp. ¶ Three rats died before end of exp. ** These groups were not run at same time as other groups in Exp. 2 but with Exp. 3.

known to affect liver fat deposition, the plan of Exp. 1 was repeated in Exp. 2 with supplements of cystine alone, or methionine and tryptophan together, added to basal diet. The level of casein in basal diet was increased to 9% and cellulose was omitted. Groups receiving 0.15% and 0.30% of choline chloride in Exp. 2 were run at the same time as Exp. 3 and not simultaneously with the other groups in Exp. 2.

When no supplement was added to the diet, liver fat results were similar to those in Exp. 1. The group which received butter fat without choline, grew at a more rapid rate than the group which received corn oil without choline, as had been observed in Exp. 1.

Supplements of methionine and tryptophan caused marked growth response. Liver fat values within the normal range were obtained in the group receiving 0.12% of choline chloride with corn oil and in the group receiving 0.15% of choline chloride with butter fat. Groups receiving 0.12%, 0.04%, and no choline chloride with butter fat, grew more rapidly than similar groups receiving corn oil. Groups which received either 0.15% or 0.30% of choline chloride were not run at the same time and there was no difference in growth between those receiving butter fat and those receiving corn oil.

The cystine supplement alone did not increase growth rate of rats. With no choline added to the diet containing cystine, 3 rats died in the group receiving corn oil and 2 died in the group receiving butter fat. These deaths occurred on 9th and 10th days of experiment. Growth rates and levels of liver fat could be calculated only for surviving rats in each group and therefore may not be representative. This is probably the reason that the group receiving butter fat did not appear to grow more rapidly than the group receiving corn oil, for only 3 rats which were growing at the most rapid rate survived in this group. Liver fat values for groups which received cystine, corn oil and no choline or

0.04% of choline chloride were higher than those for groups which received similar diets without the amino acid supplement. There was no effect of cystine on the level of liver fat in groups receiving butter fat with these two levels of choline chloride. However, cystine caused an increase in level of liver fat in the group receiving butter fat and 0.12% of choline chloride. There was no difference in level of liver fat between groups which received corn oil and those which received butter fat when diet contained no choline and cystine. In this case liver fat values for both groups were very high. With 0.04% of choline chloride, butter fat promoted a significantly higher level of liver fat than corn oil. Also levels of liver fat within the normal range were obtained with the same levels of choline as in Exp. 1 (corn oil, 0.12% of choline chloride and butter fat, 0.15% of choline chloride).

When cystine, methionine, and tryptophan were included in the diet in Exp. 3, liver fat results were, in general, the same as when methionine and tryptophan were included in Exp. 2. All groups in this series grew at about the same rate except for the group which received corn oil and no choline, which grew at a slower rate.

Histological studies were made of sections from kidneys of 3 rats from each group in Exp. 2 which received either no choline or 0.12% of choline chloride. Rats which had greatest growth rate, smallest growth rate, and growth rate closest to the mean in each group were selected for histological study.

Kidney sections were examined for signs of kidney damage as described by Christensen(8) and Hartroft(9). Hyalin casts in tubules and areas of necrosis of tubules were observed in each group which did not receive choline. No hemorrhages were observed in sections studied. Great variation in extent of kidney damage within groups and the small number of animals which were studied from each group, made it difficult to determine which group had the most severe kidney damage. However, a positive correlation was observed between rate of growth of rats and extent of kidney damage. Rats which grew at poorest rates had very few normal tubules; and, while rats that grew at intermediate rates showed considerable damage, they also had a number of normal tubules remaining. Rats that grew at the most rapid rate had very little kidney damage. This suggests that growth depressions that occurred when choline was omitted from diets were an indication of extent of kidney damage. These growth depressions were much greater when corn oil was fed than when butter fat was fed.

Discussion. If the "requirement" for choline is taken as that level of choline which is necessary to reduce level of liver fat to a minimum, results of this study indicate that rats fed on diets containing 30% of butter fat have a higher "requirement" for choline than those receiving 30% of corn oil. The "requirement" with corn oil diet was not greater than 0.12% of choline chloride and was about 0.15% of choline chloride with butter fat diet. These values were not altered when the diets were supplemented with certain amino acids although growth rates and levels of liver fat were changed in some cases.

Supplements of cystine caused increases in level of liver fat in rats fed on diets low in choline and sulfur amino acids(10). It has been suggested that increased liver fat deposition is a result of improved rate of growth caused by cystine supplement(11). In this study the cystine supplement caused increases in level of liver fat of groups which received corn oil with either no choline or 0.04% of choline chloride. With butter fat diets the cystine supplements had no effect on level of liver fat except with 0.12% choline chloride where there was an increase. Since there were no growth responses to cystine supplements, the increases in liver fat can not be explained on this basis. No conclusion concerning the effect of methionine can be drawn from these experiments.

Choline deficiency causes a characteristic kidney damage in the rat. Gyorgy and Goldblatt(12) observed that kidney damage in rats receiving no dietary choline was more severe when the diet contained hydrogenated vegetable oil than when it contained lard. If the ratio of saturated to unsaturated fatty acids in the fat is the important factor in kidney damage the corn oil used in this study

would be expected to have the same effect as hydrogenated vegetable oil used by Gyorgy and Goldblatt and the butter fat to have the same effect as lard that they used. This was found to be true in the effects of these fats on liver fat deposition(3).

Summary. 1. Rats fed on diet containing butter fat required a higher level of choline to reduce the level of liver fat to within the normal range, than did rats fed the same level of corn oil. This "requirement" for choline, as measured by liver fat, was not greater than 0.12% of choline chloride with diet containing 30% of corn oil and about 0.15% of choline chloride with diet containing 30% of butter fat. These values were not affected by supplements of cystine, or methionine and tryptophan or all 3 amino acids. 2. When no choline was fed, rats receiving butter fat grew at a more rapid rate than those receiving corn oil. It is suggested that the reduced rate of growth of rats fed corn oil was caused by kidney damage.

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A Technic for Cross-Transfusion of Blood in Embryonic Chicks and Its Effect upon Hatchability.* (22868)

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Although several methods for intravenous injection of chick embryos are described in the literature(1-5), none were used for purposes which required the chick to hatch after injection or withdrawal of blood. In the course of our studies on the homograft problem, it became necessary to cross-transfuse blood between 2 embryonic chicks for subsequent testing of skin graft compatibility after hatching. Trials of presently known methods for intravenous injection of chick embryos did not lead to significant survival through hatching. Most of recent technics consist essentially of locating the vessel to be injected by candling, cutting a window in the shell, putting a drop of mineral oil on the shell membrane to make the vessel visible, injecting or withdrawing blood with a 26-27 gauge needle on a syringe, withdrawing the needle, and sealing. Although this procedure seems simple, we have encountered two principal difficulties. Firstly, it is difficult to hold the needle in a small vessel for any length of time —especially if considerable amounts of blood must be withdrawn. In an attempt to solve this problem, Goldwasser and Shelesnyak(5) constructed an U-shaped egg clamp and an elaborate syringe carrier which was movable in 3 dimensions by gears. The advantage of such a stable apparatus, however, is offset by its complexity and cost of construction. The second and most important difficulty is con-

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trol of hemorrhage after withdrawal of needle. Bleeding, of course, is greater the younger the embryo, since the clotting mechanism develops gradually. The effect of extensive hemorrhage becomes more noticeable when hatching of chicks is desired, since loss of blood may not be fatal immediately, but may show its effects later in development. In previous studies designed primarily for short term experiments, bleeding has been controlled in various ways: by selecting small veins for injection(4), clamping for a short period(4), withdrawing the needle slowly(2,3,5) or cauterization(1). We have tested these methods and found that none of them results in more than approximately 40% hatching. The following method has thus far yielded 82% hatching in more than 500 eggs tested.

Method. Equal numbers of White Leghorn and New Hampshire eggs were used. The eggs were put in incubator, blunt end up, and turned every 2 hours on automatic turning trays until 7th-9th days of incubation. The eggs were then incubated on their sides 2-4 days to move the main allantoic veins to the side, and uppermost side was marked. The main trunk of the allantoic vein, where chorioallantoic veins converge and enter the embryo, was then located. It is usually the largest vessel visible, and by moving the egg back and forth under a bright candler, its attachment to the embryo and to the shell can be found. The location of the vessel and direction of flow in vessel, *i.e.* toward the embryo, was marked on the eggs. A window in the shell approximately 1 x 1.5 cm was then cut with abrasive disc on a motor; care was taken not to nick the shell membrane. To stiffen the shell membrane and loosen it from the shell, mineral oil was applied to the cut edges. The shell was then taken off with a sharp needle probe and paraffin or scotch tape put over the window until the egg was ready for operation. For injection or withdrawal of blood, paraffin or scotch tape over shell membrane was taken off, and a drop of sterile mineral oil was put on window to make the vessel visible. The egg was then put on a clay mold with layer of gauze put between egg and clay to prevent adherence of excess oil on the surface of the shell. On this flexible mold, the egg was guided so that the vessel was pierced in the direction of blood flow with a 30 gauge hypodermic needle mounted on a camera tripod head (Fig. 1). To prevent any air from entering under the shell membrane, thus pushing the chorioallantoic membrane down, a drop of paraffin was immediately placed over the point of needle entry (Fig. 2). The overall apparatus for crossing blood between two eggs is shown in Fig. 3. The polyethylene tubes with the 3-way stopcock and hypodermic needle shafts were previously sterilized by soaking in aqueous solution of zephiran chloride for 24 hours. Heparinized saline was used in syringe and tubes to prevent clotting, to provide a fluid column for smooth injection or withdrawal, and to make cleaning of tubes after each injection easier. Fifteen to 40 units of heparin/ml of saline were used for 9-12-day embryos, and 50-100 units/ml for 14-18-day embryos. To prevent any intermixture of blood and saline, an air bubble was always kept between the two fluids. Any inequality in amount of blood drawn up in the 2 tubes was controlled by the three-way valve. Cross injections of blood from one egg to the other were made by detaching the polyethylene tube from the rigidly held needle (Fig. 4) and hooking it onto the other needle. Upon cross-exchange of blood, paraffin was put on the window and around the needle to hold it in place, labelled with tag, and a stylet was put in lumen of needle to make subsequent cleaning easier (Fig. 5). The egg, with needle attached, was removed from needle-holder (Fig. 6), and returned to incubator in the same position that it held during the previous 3-4 days; thus, no shearing of the needle from vessel by movement of chorioallantoic vessels could take place. In the case of 9-11-day incubated eggs, the needle was withdrawn from the vessel on 14th day, and the paraffin around the needle was pressed over the opening. In 16-18-day eggs, the needle was withdrawn 1-2 days after operation. Subsequently, the eggs were incubated in the automatic turning trays with air sac end up until 18th day, when they were put on their sides in another tray divided into

The egg is guided so that the vein is piereed by the needle (Fig. 1), and melted paraffin immediately put on shell membrane at point of needle entry to prevent entrance of air (Fig. 2). Fig. 3 shows overall apparatus when cross-transfusing blood. After withdrawal of blood the polyethylene tubes are unhooked and put on the other needle for inj. (Fig. 4). The egg is then tagged and a stylet put in the lumen of needle (Fig. 5). Finally, the egg with the needle attached is taken off the needle-holder (Fig. 6).

separate compartments. To test another method of controlling bleeding, cautery of blood vessels, with and without .04 ml of blood cross transfer, was done on 9-13-day embryos. Blood was injected and withdrawn as described. The shell membrane was then taken off, and the vessel cauterized as the needle was withdrawn. To seal the window, previously sterilized "saran wrap" plastic was laid over it and melted paraffin placed around the edges. To test the effect of transference of the air sac, the shell membrane was torn, and the window sealed as in the cauterized eggs.

Results. As is shown in Table I, 87% of 408 untreated control eggs, that were viable on 9th-11th day of incubation, hatched. Of 567 eggs with .04-0.4 ml of blood cross exchanged at 9-18 days of incubation, 82% hatched. Thus, with our method there was almost no effect on normal percentage of hatch, although 3% of hatched chicks were

TABLE I. Effect of Intravenous Cross-Transfusion during Embryonic Stages upon Hatchability of Chicks.

ml of blood cross-transfused	Incubation, days	No. hatched/No. done	No. aborted at: 18-21 days incubation	14-18 days incubation	10-14 days incubation
.04	9	29/ 34	5	0	0
	10	52/ 68	11	3	2
	11	24/ 29	2	1	2
	Subtotal	105/131 (80%)	18 (14%)	4 (3%)	4 (3%)
.1	10	26/ 30	4	0	1
	11	11/ 16	2	0	3
	13	6/ 9	2	1	0
	Subtotal	43/ 55 (78%)	8 (14%)	1 (2%)	4 (7%)
.1 (twice)	10	19/ 21 (90%)	2 (10%)	0	0
.2	10	15/ 18	3	0	0
	11	127/148	13	2	6
	12	8/ 8	0	0	0
	18	8/ 9	1	0	0
	Subtotal	158/183 (86%)	17 (9%)	2 (1%)	6 (3%)
.2 (twice)	10	19/ 21	2	0	0
	11	76/ 97	14	2	5
	16	13/ 17	4	1	0
	Subtotal	108/135 (80%)	20 (15%)	3 (2%)	5 (4%)
.4	14	16/ 21	1	1	3
	16	10/ 11	0	1	0
	17	7/ 10	2	1	0
	Subtotal	33/ 42 (79%)	3 (7%)	3 (7%)	3 (7%)
	TOTAL	466/567 (82%)	68 (12%)	13 (2%)	22 (4%)
Controls—No previous treatment		355/408 (87%)			
Inj. into arteries		16/ 19 (84%)	3 (16%)	0	0
Cautery of vein, with or without blood transfer		23/ 64 (36%)	21 (33%)	3 (5%)	17 (27%)
Air sac transferred to side on 11th day		19/ 32 (59%)			
Air sac transferred to side on 17th day		21/ 26 (81%)			

lame or otherwise sickly at hatching. All other chicks were healthy, showing no outward effects of the blood transfer. Over 200 of these chicks have been kept and observed for 3-9 months. Of the aborted eggs, most deaths occurred shortly before hatching (12%). Some deaths occurred after 14-18 days incubation (2%), and some shortly after cross injection (4%).

It should be mentioned that these eggs represent the results of 34 consecutive separate experiments since the development of this technic. Eggs of only one experiment were not included in the Table. Due to accidental failure of incubator humidity control, the humidity dropped to 47% when the eggs had been incubated 11-13 days. In this experiment, 9 out of 11 died shortly after the operation and only one hatched.

The percentage of chicks hatched did not vary significantly with withdrawal and injection of .04-0.2 ml of blood in eggs incubated 9-18 days. Nor did the per cent mortality differ with the cross-transfer of 0.4 ml of blood in 14-17-day-old eggs. The injection and withdrawal of 0.2 ml of blood once and twice, resulted in 86% and 80% hatch rate, respectively. Also, as can be seen in Table I, incubation age at which blood is cross-transferred (between 9th and 18th day) does not seem to affect hatching in any significant way.

Insertion of needle into arteries had very

little effect on hatchability of eggs, since 16 out of 19 (84%) hatched. However, of the 16 hatched, 5 (31%) were lame. As mentioned previously, when the needle was inserted, presumably in the direction of flow into veins, only 3% were lame. It is possible that these lame chicks may have resulted from inadvertent injection in opposition to the flow in the vein.

Withdrawal of the needle just after injection, followed by cautery of blood vessels, produced only 36% hatch in the 64 eggs tested (Table I). About a quarter of the eggs so tested died shortly after cauterization, and a third died just before hatching. Because cauterization involves removing the shell membrane and consequent shifting of air sac to an abnormal region, the effect of tearing the membrane was tested on eggs. As shown in Table I, when this procedure was used, 59% of the eggs operated upon on the 11th day hatched, thus accounting for much of the mortality due to cautery. Tearing the shell membrane on 17th day of incubation, however, had hardly any effect since 81% of these eggs hatched.

Discussion. With the present technics, 82% of intravenously cross-transfused chick embryos have hatched as compared to 87% of controls. The advantages of this technic as far as obtaining a large percentage of hatchability are the following: 1) Almost no bleeding occurs. This has been accomplished by leaving the needle in the vessel until clotting mechanisms of the blood are more fully developed and until the vessel tended to produce scar tissue around the needle. Other methods of controlling bleeding have usually depended upon selection of small vessels, but when fairly large amounts of blood must be withdrawn, the task of withdrawing blood from small, thin-walled vessels is a tedious one. If medium or large-sized vessels were used, clamping of vessels or slow withdrawal of needle did not prevent hemorrhage. Cauterization of vessels produced only 36% hatchability. 2) The rigid needle support makes piercing of blood vessel relatively simple. It also keeps the needle stable, thus permitting slow injection or withdrawals. 3) For cross-transfusion of blood, the vessel need be pierced only once both for removal and injection of blood. 4) By not removing the shell membrane, the air sac is left intact. Leaving the air sac in its natural position at the blunt end seems to be important for the normal hatching of eggs. If the air sac was transferred to the side on 10th-11th day of incubation only 59% hatched as compared to 87% of control eggs. 5) A minimum of sterile technic is necessary, since the contents of the egg are not exposed to the exterior except at point where needle is inserted through the shell membrane. 6) The method is a fairly rapid one; the cross-transfusion of 0.2 ml of blood between 2 eggs can be done in 5-10 minutes. 7) The method permits multiple bleeding or injection during the course of incubation, since the needle is not removed, and clotting in lumen of needle is prevented by the stylet.

For eggs earlier than the 8th day of incubation, preliminary trials indicate that the method must be somewhat revised since the yolk sac vessels are more mobile and less firmly attached to the shell membrane. After 17th day of incubation the eggs are also difficult to work with due to development of vasoconstriction mechanisms which cause the vessels to constrict upon contact with the needle. It is important in these advanced stages to inject the large main trunk of the allantoic vein.

Summary. A method is described by which .04-0.4 ml of blood may be cross-transfused in 9-18-day-old chick embryos with almost no increase in mortality over untreated control eggs. Of the 567 eggs tested, 82% hatched, as compared to 87% of the control eggs.

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Rate of Glycogenesis in Liver of Depancreatized Rat after Parenteral Administration of Glucose and Fructose.* (22869)

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Previous work(1) has shown that fructose forms glycogen in the liver faster, and to a greater degree, than does glucose when fructose and glucose are injected intraperitoneally into normal fasted rats. These findings suggested that it would be of interest to extend the same method of studying glycogenesis to totally depancreatized rat following administration of these sugars. Treadwell and Roe, Jr., published a procedure for total pancreatectomy of the rat(2). When evaluated by histological studies, determination of amylase content of areas which the pancreas normally occupies, and glucose tolerance tests, it was found that this operation removes essentially all of the pancreas. This operative procedure was used for the studies reported. Since it was desired to study the effect of absence of insulin on glycogenesis in a large number of rats, it was important to establish criteria of the presence of marked diabetes. Only animals conforming to the following criteria were placed in the experiment: (1) Polyuria (20 to 50 cc of urine/day) in fed rats; (2) Glucosuria in fed rats, as indicated by a 3 or 4 plus Benedict's qualitative test; (3) animals receiving fructose were given a glucose tolerance test previous to glycogenesis experiment, and sugar tolerance of animals receiving glucose was tested during the glycogenesis experiment by determining a control and a one-hour post-injection blood sugar value. Only animals were accepted for the experiment which showed a one-hour post-injection blood sugar level above 200 mg% following injection of 2 g of glucose/kg body weight. Fasting blood sugar level could not be used as a criterion of the presence of experimental diabetes. This is indicated by the data of Table I, in which are shown blood sugar levels of depancreatized rats at different intervals during a 30-hour fast. Within 18 hours after withholding food, 4 of the 6 animals had a normal blood sugar level. Animal No. 5 on other occasions showed blood sugar levels of 80 and 89 mg% after a 24-hour fast. The blood sugar of all animals returned to hyperglycemic levels in 12 hours after food was allowed again. It appears that normal blood sugar level of fasted depancreatized rat corresponds to the presence of a low liver glycogen value. In 27 depancreatized rats that had a normal blood sugar level after a 24-hour fasting period and were given saline injections, the average liver glycogen was 83 mg per 100 g of liver. Data in Table II show a lower glycogen level in depancreatized rats than in normal rats upon a Purina chow diet. In these studies the liver glycogen content was about one-fourth that in normal animals. However, some glycogen was formed in the liver in absence of the pancreas. It is suggested that these results are probably due to absence from the alimentary tract of pancreatic digestive enzymes that split carbohydrates and proteins.

Methods. White rats from the same stock as used in previous studies(1), weighing 150 to 200 g, were depancreatized. Two to 4 weeks after the operation, the animals were fasted for 24 hours. At the end of the fast they received an intraperitoneal injection of

TABLE I. Blood Sugar Level over a 30-Hour Fasting Period. Depancreatized rats were removed from Purina chow. Values expressed as mg %.

Hr of fasting	Animal No. 1	2	3	4	5	6
0	450.0	193.5	228.0	453.3	413.1	331.8
6.25	399.0	103.5	234.6	339.3	330.6	190.5
14.75	390.3	80.2	95.6	106.5	235.0	75.5
18.0	356.7	72.7	95.2	63.0	338.5	69.0
21.0	360.9	62.8	106.4	65.5	354.2	76.9
24.0	317.4	55.3	112.2	56.1	348.2	82.6
30.0	176.9	60.9	114.6	65.4	302.8	71.2
Returned to food						
12.0	485.0	215.7	344.1	463.2	444.9	343.8

*Supported by a grant from N.I.H., U.S. Public Health Service.

TABLE II. Effect of *Ad Libitum* Feeding on Liver Glycogen. Animals were removed from an ample supply of Purina chow checkers, anesthetized, and whole liver removed for analysis.

	No. of animals	Avg wt, g	Avg liver wt, g	Avg glycogen value, mg %	Avg blood sugar, mg %
Normal	5	270	11.11	3454.3	130.9
Depancreatized	5	180	8.10	895.8	392.0

10% solution of glucose or fructose at dose level of 2 g/kg body weight. At different intervals after administration, the animals were anaesthetized with nembutal and the whole liver was quickly removed and homogenized in 5% trichloroacetic acid. The glycogen was determined by the anthrone method of Carroll, Longley and Roe(3). Control animals received saline of a comparable volume and treated the same as sugar injected animals. The average liver glycogen value of saline injected controls was subtracted from average liver glycogen value of the sugar injected animals.

Results. Liver glycogen values following administration of glucose and fructose, plotted against time, are shown in Fig. 1. It can be seen that fructose forms liver glycogen in the depancreatized rat more rapidly, and to a greater extent, than does glucose under the same conditions. However, glucose

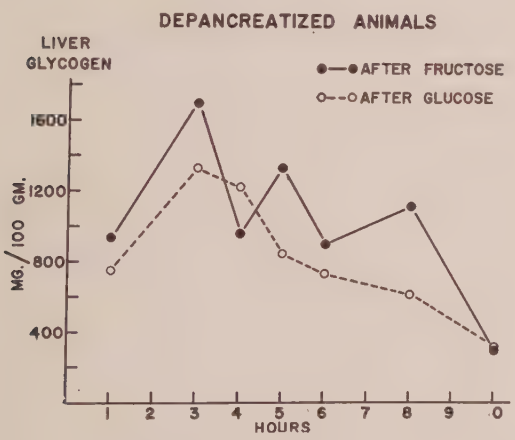

FIG. 1. Liver glycogen formation in depancreatized rats following intraper. inj. of fructose and glucose. Curves represent values from a total No. of animals as follows: fructose, 30 rats; glucose, 33 rats.

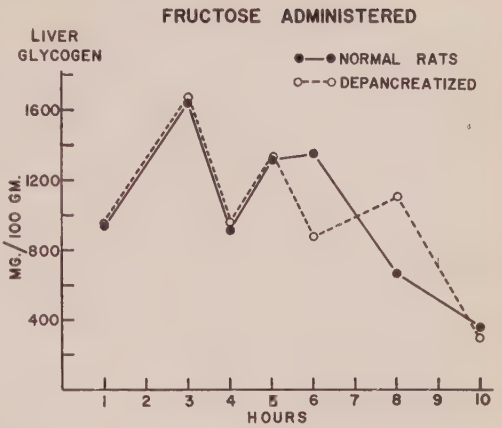

FIG. 2. Comparison of rates of glycogenesis in normal and depancreatized rats following intraper. inj. of fructose. Curves represent values from a total No. of animals as follows: normal rats, 66; depancreatized rats, 30.

does form liver glycogen in the depancreatized rat at a considerable rate.

Fig. 2 shows a comparison of response to fructose administration in the normal and the depancreatized rat. The response is strikingly similar during the first 5 hours and is essentially the same during later hours after administration. It is interesting to note that the drop in liver glycogen curve at the 4-hour point, as observed with normal rats, was repeated in the case of depancreatized animals.

Fig. 3 shows a comparison of response to glucose injection in the normal and the depancreatized rat. It can be seen that the depancreatized animals formed liver glycogen at essentially the same rate as that observed with the normal animals, when glucose was administered intraperitoneally.

Discussion. The results obtained with fructose in the depancreatized animal were not surprising in view of considerable evidence indicating that fructose utilization is not impaired in the absence of insulin(4-7).

It is generally agreed that insulin is essential for the formation of liver glycogen from glucose. It has also been claimed that insulin promotes hexokinase activity by opposing inhibition of this enzyme by the anterior pituitary(8). Our results with experimentally diabetic rats indicate that the metabolic processes in the liver leading to formation of

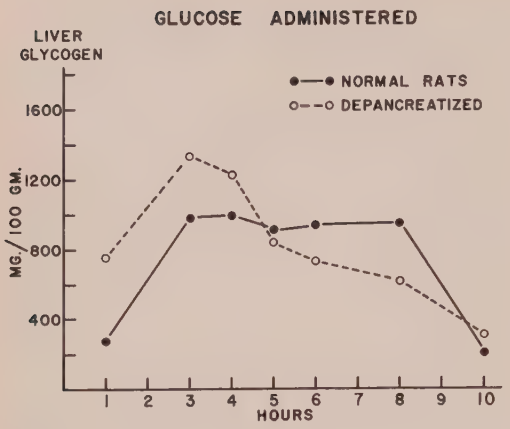

FIG. 3. Comparison of rates of glycogenesis in normal and depancreatized rats following intraper. inj. of glucose. Curves represent values from a total No. of animals as follows: normal rats, 46; depancreatized rats, 33.

glycogen are not directly influenced by insulin. In fact, our studies lead to the suggestion that insulin may have an effect on glycogenesis which is contrary to the generally accepted view, namely, it may decrease the rate of glycogenesis in the liver by diminishing the level of hyperglycemia that results from administered glucose. This interpretation seems applicable since an increase in blood sugar to hyperglycemic levels is a stimulus to glycogenesis, though the blood sugar level is probably not the only determining factor. This view is supported by the work of Cori, who found that injection of insulin into normal rats markedly diminished formation of glycogen in the liver following oral administration of glucose and fructose(9).

Summary. 1. Following intraperitoneal injection, fructose formed liver glycogen more rapidly, and to a greater extent, than glucose in depancreatized rats. 2. When injected intraperitoneally, glucose formed liver glycogen as rapidly in depancreatized rats as in normal rats. 3. Absence of the pancreas from the otherwise intact rat showed no apparent effect upon activity of any of the enzymes involved in formation of glycogen from fructose or glucose in the liver. 4. The data obtained suggest that the metabolic processes in the liver involved in formation of glycogen from fructose or glucose are not directly influenced by insulin.

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X-Ray Irradiation of Pregnant Rats: Attempts to Produce Congenital Cardiac Anomalies in Offspring. (22870)

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X-ray irradiation of pregnant animals has long been known to induce various malformations in the offspring of the animals thus treated. Certain types of deformities have been shown to result from single doses of x-rays given at specific stages of pregnancy (1,2). No changes in the heart, however, have been reported to follow this treatment. The lack of information on this point may be due to one of several reasons, among which may be the high resistance of this organ or the occurrence of cardiac lesions which cause intra-uterine death. Still another possibility is that the changes may have been overlooked, because of technical difficulties, in examination of the offspring. Our work was performed primarily with a view toward overcoming the last possibility and secondarily, because of the need for an experimental method of producing changes resembling the congenital cardiac anomalies in man.

Methods. Pregnant rats were treated with one irradiation each. Pregnancy was considered to prevail when spermatozoa were found in the vagina in the daily examination of the rats. The newborn rats were allowed to live for at least 10 days. Necropsy was done under a dissection microscope, with a magnification of 7 times. After the front portion of the thorax had been removed, the heart and the left lung were pushed to the animal's right. The aorta was inspected from the diaphragm to the ligamentum arteriosum, which was then cut, as was the left superior vena cava. The latter is a normal structure in the rat. The heart was then opened in the following order: 1. The right atrium from the inferior vena cava to the right superior vena cava. 2. The tricuspid valve and the right ventricle along its lateral border to the apex. From the apex the anterior wall of the right ventricle was opened ventrally through the outflow tract and the incision was carried into the pulmonary trunk. 3. The left atrium along the lateral border. 4. The left ventricle from the mitral valve to the apex along the lateral edge, and farther from this edge ventrally through the aortic valve. The fact that the rats had been allowed to reach a development of 10 days allowed a good view of the thoracic aorta, the ligamentum arteriosum, the atrial septum (from the right atrium), the ventricular septum (from the right ventricle and, especially below the aortic valve, from the left) and of each valve. After the left atrium had been opened, the orifices of the 4 pulmonary veins could be identified.

Results. The number of treated animals and of newborn rats, as well as the doses used, are shown in Table I. It is evident that a large number were treated with doses too high to permit survival of the young. Results were obtained from 46 young rats which lived for 10 days and which had been born to mature females irradiated on the sixth to the sixteenth day of pregnancy. The findings in the heart and great vessels in the thorax were consistently normal. This of course does not mean that cardiac lesions in viable rats cannot be obtained in this way, but the expected percentage of these instances is low among rats surviving 10 days. Some of those dying earlier may have had malformations but were not examined. The above results pertain particularly to radiation on the sixth and thirteenth days when adequate numbers of animals were used.

Summary. Pregnant rats were treated with single doses of x-ray irradiation. The heart and great vessels in thorax of offspring were investigated when the rats had reached at least 10 days of age. The method of dis-

* The Mayo Foundation, Rochester, Minn., is a part of the Graduate School of University of Minnesota.

TABLE I. Doses, Number of Female Rats Irradiated and Resulting Offspring.

Doses in r/rat	No.	Day after insemination 2	4	5	6	7	8	9	10	11	12	13	14	15	16	18
400	R.t.*	1	1		2	3	2	2	2	2	2	1	2			
	N†	0	0		0	0	0	0	0	0	0	0	0			
300	R.t.						1	1	2	1	1	3	1	1	1	1
	N						0	0	0	0	0	0	0	2(0)	0	10(0)
250	R.t.						1	1	1							
	N						0	0	0							
200	R.t.				1	1	1	2	2	2	2	1	1			
	N				5(3)	0	1(0)	0	1(0)	0	0	0	0			
125	R.t.	1	1	1	1	1	2	1	1	1	1	1	1			
	N	0	0	0	11(9)	0	2(0)	0	1(0)	0	0	2(0)	0			
75	R.t.						2	1	2	2	2	1	3		1	
	N						8(3)	8(2)	6(5)	3(1)	2(1)	14(12)	7(4)		8(6)	
Total No. newborn		0	0	0	16(12)	0	11(3)	8(2)	8(5)	3(1)	2(1)	16(12)	7(4)	2(0)	8(6)	10(0)

* R.t. = Rats treated. † N = Newborn; stillborn not included.
Figures within parentheses give No. of newborn living 10 days or more.

section is described. In 46 young rats that survived 10 days no cardiac abnormalities were found. Examination of young rats that died before 10 days was not carried out.

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Oxygen Tension of Subcutaneous Gas Pockets in Cobalt-Treated Mice and Adrenalectomized Mice.* (22871)

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Cobalt (II) chloride† and adrenalectomy (2) have both been shown by Gerschman *et al.* to give protection against the toxicity of oxygen at high pressures in mice. The experiments described below were undertaken to see if these two treatments furnish their protection by causing a generalized decrease of tissue pO_2, such as might result from a circulatory or respiratory response to the treatment.

Campbell(3) was the main proponent of utilization of subcutaneous or intraperitoneal gas pocket containing nitrogen as index of tissue tension, and the method has been applied in studies of oxygen at high pressures by Campbell(4,5,6,7), Sibree(8), Taylor(9,10), Lambertsen *et al.*(11) and Bahnson and Matthews(12).

Methods. Subcutaneous gas pockets were formed in 20 g female mice of Carworth Farms Webster strain, by injecting 4 to 8 cc of air or a gas mixture (7% CO_2, 7% O_2 in N_2) under the skin of the back. No anesthetic was used. It was assumed that after a day, the oxygen tension in the pocket had come to a steady state level which was within 2 mm of the mean tension in the surrounding tissues. To sample, approximately 2 cc of

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† Unpublished observations of Gerschman, R., and Gilbert, D. L.

the pocket gas were withdrawn into a syringe, the dead space of which had been filled with dilute acid solution. After sampling, the mouse's pocket was refilled by injection of air or gas mixture. Analysis for percentage CO_2 and O_2 were made on a Scholander Micro Gas analyzer. Percentages were converted to partial pressures on the assumption that the total pressure in the pocket was one atmosphere. The work included 3 series: (a) effect of previous injections of $CoCl_2$ on mice submitted to one atmosphere of O_2 and on mice breathing room air, (b) time course of cobalt effect, and (c) effect of adrenalectomy. (A) In the first series, 9 mice were given 10 daily intraperitoneal injections of .0012 mM $CoCl_2$ in a volume of 0.2 ml, and 9 mice were given 10 daily IP injections of 0.2 ml of 0.9% NaCl. On the day of tenth injection, all mice were given subcutaneous gas pockets. The next day, the pockets were sampled and then 5 mice of each group were placed in a chamber containing 100% O_2 at 1 atmosphere. The rest of the mice were left in room air. The pocket gas compositions were analyzed each day in all 4 subgroups until the mice in the O_2 chamber had died. (B)

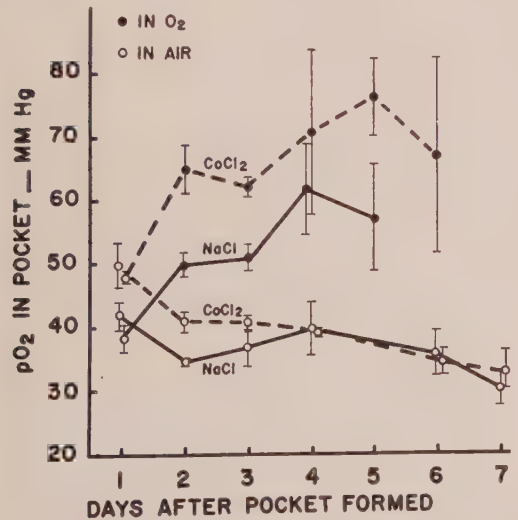

FIG. 1. Oxygen tension of subcut. tissue in mice as measured by the gas pocket method. Points are mean values and vertical lines represent stand. errors. ○, animals breathing air; ●, animal breathing O_2 at 1 atmosphere. For 10 days prior to day zero, 2 subgroups were inj. with $CoCl_2$ (– – – –) and 2 subgroups were inj. with saline (———). Pockets were formed on day zero.

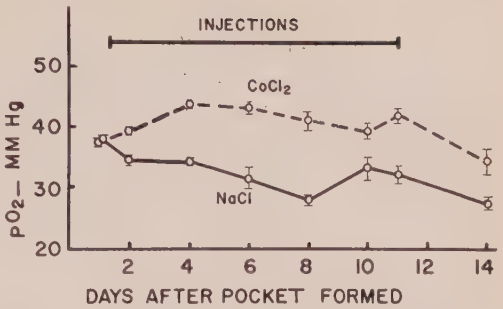

FIG. 2. Oxygen tension of subcut. tissue in air-breathing mice as measured by gas pockets during 10 daily inj. of $CoCl_2$ (– – – –) and of saline (———). Points are mean values and vertical lines represent stand. errors. Pockets were formed on day zero.

In the second series, 2 groups of 5 mice were given subcutaneous pockets. The next day, the pockets were sampled and then one group was started on a regimen of 10 daily IP injections of .0012 mM $CoCl_2$ and the other group started on 10 daily IP injections of 0.2 ml of 0.9% NaCl. Pocket O_2 tensions were analyzed throughout the injection period. (C) Gas pockets were formed in a group of 5 intact control mice and in 5 animals which had been adrenalectomized 5 days previously. Samples for gas analysis were taken 1 day after pocket formation and again a week later.

Results. Fig. 1 shows the results of the first series of $CoCl_2$ injections. The points on the graph are mean values and the vertical lines indicate standard errors. It is seen that the cobalt-injected mice had higher pocket O_2 tensions both in O_2-breathing and air-breathing animals during the first 3 days.

The second series of cobalt injections is shown in Fig. 2. There was a definite increase in pO_2 in the cobalt-treated mice after the first injection. After the second injection, the difference was about 10 mm, and this difference was maintained for the rest of the experiment.

The adrenalectomized mice had a mean pO_2 of 31.6 ± 0.6 (standard error) in a day-old pocket and 32.3 ± 5.8 in a week-old pocket; the intact control animals had a mean pO_2 of 33.6 ± 1.7 in one day and 30.8 ± 1.2 in one week. Therefore, adrenalectomy did not seem to have any significant effect on the pocket pO_2.

Discussion. It was found that when the mice were disturbed or handled in sampling, the pCO_2 of their gas pockets fell rapidly although the pO_2 remained nearly constant for as long as 10 minutes. Therefore, CO_2 analyses are not reported, since it was felt that only the O_2 values were accurate estimates of tissue tension of the undisturbed mice.

The data shown in Fig. 1 are consistent with the work of other investigators who analyzed gas pockets in animals subjected to a high percentage of O_2 at one atmosphere (4,5,6,8,9,12). The increase of pocket pO_2 on going from air to O_2 is small (about 20 mm) compared to the change of inspired pO_2 (590 mm). The pocket tensions in an O_2-breathing animal are fairly uniform until the last hours of the animal's life, when the pocket pO_2 falls and the pCO_2 rises. Samples were obtained from 3 of the O_2-breathing mice just before death:

Min. before death	pCO_2	pO_2
31	80	50 mm Hg
2	71	6
1	108	10

The CO_2 tensions are higher than 70 mm, the maximum found during the first 3 days in O_2 before terminal changes could be expected. Also, it is seen by the low pO_2 that the animal's death is accompanied by tissue anoxia, probably due to respiratory or circulatory collapse, or both.

Fig. 1 and 2 show that at least in the tissue surrounding the subcutaneous pocket, cobalt injections are associated with a pO_2 which is higher than normal, and the analyses in adrenalectomized mice indicate no change of pocket pO_2. These data are evidence against the hypothesis that the protective effect in O_2 poisoning is due to a general respiratory and/or circulatory depression which would deliver less O_2 to the tissues. However, there is still the possibility that blood may be shunted away from sensitive organs.

Several factors can be mentioned as possible explanations of the cobalt results: (a) Cobalt is a well-known hematopoietic agent so that the increased tissue pO_2 might be due to an increased carrying capacity of the blood for O_2. However, 5 cobalt-injected mice had a mean hematocrit ratio of 54 ± 2 (standard error) compared with 5 saline-injected animals who had a mean ratio of 51 ± 1 after 10 days of treatment; Gessert and Phillips(13) showed no significant cobalt-induced polycythemia in rats until after 10 days. Fig. 2 shows that there is a definite effect on the pocket pO_2 after only one injection so that polycythemia can probably be excluded as a factor. (b) It was noticed during these experiments that cobalt-injected mice were easily excited and more irritable than the normals, so the increased pO_2 could be caused by hyperventilation and increased cardiac output due to the chronic excitement of the animal. Why cobalt should cause an increased irritability remains to be explained. Such an irritability is not mentioned in reports of cobalt administration to human subjects where it would probably be easily noticed(14). (c) There is evidence that cobalt can cause a disturbance in regulation of blood sugar(15,16), but how this could be connected with tissue pO_2 is not clear. (d) Finally, cobalt is known to act as an enzyme inhibitor in some oxidative processes(17) so it is possible that decreased utilization of O_2 might explain in part the elevated pO_2.

It has often been suggested that cobalt leads to a histotoxic anoxia which stimulates erythropoiesis(18). If the increased pO_2 found in subcutaneous pockets of cobalt-treated mice can be generalized to the whole body, it is seen that there is an abundant supply of oxygen to the tissues, and any disturbance of oxidation must indeed be found at the cellular or enzyme level.

Summary. The gas pocket method of estimating O_2 tension of subcutaneous tissue was utilized to see if the protection afforded by $CoCl_2$ and adrenalectomy in high oxygen pressure could be due to a generalized depression of tissue pO_2. (a) Mice previously treated with cobalt and control animals previously treated with saline were studied in O_2 at one atmosphere and in room air. (b) Pockets were observed in an experimental group during a period of cobalt injections and

in control animals which received saline injections. (c) Gas pockets in a group of adrenalectomized mice were compared with those in intact, untreated controls. The cobalt injections produced an increase in pO_2 of the gas pocket which appeared the first day after beginning treatment. The adrenalectomized mice showed no difference from their controls in gas pocket pO_2. Since decreased pO_2 was not observed, it was concluded that the protection of the two treatments against oxygen toxicity must be due to other factors.

The author is greatly indebted to Dr. R. Gerschman, Dr. D. L. Gilbert, Dr. W. O. Fenn and Mr. D. Caccamise for help in this work.

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Abolition by Hypophysectomy of the Anticortisol Action of Desoxycorticosterone. (22872)

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Numerous experiments have shown that there is an antagonism between glucocorticoids and mineralocorticoids, as regards their effects upon inflammation, in that the inhibitory action of the former can be blocked by the latter, under suitable experimental conditions. However, these opposing effects appear to be exerted through different mechanisms. The anti-inflammatory action is presumably direct, since it occurs even topically, at the site where glucocorticoid (*e.g.* cortisol) is applied, while the contrary effect of mineralocorticoids (*e.g.* desoxycorticosterone) is probably indirect because it can be obtained only by systemic treatment. An essential difference in modes of action of these two types of corticoids is also suggested by the observation that hypophysectomy does not interfere with most of the classic glucocorticoid effects, but does prevent many of the morbid lesions characteristic of mineralocorticoid overdosage, such as nephrosclerosis, periarteritis nodosa and hypertension(1,2,3,4,5,6).

In view of these facts it appeared of interest to determine whether, even in the absence of the hypophysis, systemic treatment with a mineralocorticoid would exert pro-inflammatory actions in itself, or block the anti-inflammatory effect of a glucocorticoid. It had been demonstrated that the "granuloma-pouch" technic lends itself well for quantitative assessment of inflammation in hypophysectomized rats(7) and, therefore, we used this test object for our studies.

Materials and technics. Eighty female Sprague-Dawley rats, with mean initial bodyweight of 160 g (range: 150-168 g), were subdivided into 7 groups, as indicated in Table I. On the first day, a granuloma-pouch was produced as follows: (1) injection

TABLE I. Somatic and Organ-Weights in the Hypophysectomized Rat.

Group	Treatment	No. of animals	Final body wt, g	Wt loss, %	Exudate, ml	Granuloma pouch, mg	Thymus, mg	Spleen, mg
1	Hyp-X	10	138 ± 4.9	14	18.7 ± 1.8	2442 ± 246	149 ± 16	290 ± 16
2	" + COL-Ac	10	114 ± 1.5	29	8 ± 1.2	1357 ± 115	28 ± 1.6	179 ± 9.1
3	" + DOC-Ac	10	137 ± 4.4	16	18.8 ± 1.8	2438 ± 198	117 ± 18	262 ± 18
4	Hyp-X + COL-Ac + DOC-Ac	10	114 ± 1.8	29	8.6 ± 1.3	1408 ± 113	13 ± 5.4	181 ± 8.2
5	Hyp-X + Adr-X + COL-Ac	13	117 ± 1.4	27	4 ± .74	933 ± 104	216 ± 2.5	161 ± 14
6	Hyp-X + Adr-X + DOC-Ac	14	138 ± 3	15	18.5 ± .7	2712 ± 179	128 ± 14	279 ± 15
7	Hyp-X + Adr-X + COL-Ac + DOC-Ac	13	123 ± 1.3	23	7.1 ± 1.1	1196 ± 160	28 ± 2.2	191 ± 7.7

through a No. 27 gauge needle of 25 ml of air into subcutaneous tissue underneath the shaved dorsal skin, to form a single cavity, (2) without withdrawing the needle 1 ml of 1% croton-oil solution (in corn oil) was injected into the air sac thus created. Forty-eight hours later the animals of all groups were hypophysectomized through the parapharyngeal route and those of Groups 5-7 were bilaterally adrenalectomized through the lumbar route. At that same time, treatment was begun with cortisol acetate (COL-Ac) with 400 μg/day alone or with desoxycorticosterone acetate (DOC-Ac) at daily dose of 100 μg, both compounds being injected subcutaneously in the form of microcrystal suspensions in 0.2 ml of water. All animals were allowed free access to Purina Fox Chow and Pablum. Four rats each in Groups 1 and 3, one rat each in Groups 4 and 5 and three rats in Group 6 died during the experiment. All the remaining animals were killed on 12th day after initiation of hormone treatment. Immediately afterwards the exudate was measured by aspiration into a graduated syringe, while the granulomatous walls of the pouches and the organs listed in Table I were dissected and fixed in Susa solution for subsequent weighing.

Results. Table I summarizes our principal results. Although all animals lost some *body-weight,* those both adrenalectomized and non-adrenalectomized and given COL-Ac exhibited the most pronounced catabolism. This weight loss was not inhibited by concurrent treatment with DOC-Ac, either in the merely hypophysectomized or in the hypophysectomized-adrenalectomized animals (Groups 4 and 7). *Exudate formation* and weights of *granuloma-pouches* were significantly depressed in all groups receiving COL-Ac. This anti-inflammatory effect was more pronounced in animals which were both hypophysectomized and adrenalectomized (Group 5) than in those merely hypophysectomized (Group 2), since the apparent difference in exudate formation ($P<0.02$), and in weight of granuloma-pouches ($P<0.05$) was significant. It is doubtful, however, whether this small difference should be ascribed to the anticortical effect of endogenous mineralocorticoids secreted by adrenals of the merely hypophysectomized rats; possibly the animals subjected to the double operation were just too damaged to respond normally. In any event, concurrent treatment with DOC-Ac did not significantly inhibit the anti-inflammatory action of COL-Ac, either in the merely hypophysectomized or in the hypophysectomized and adrenalectomized rats. Similarly, the *thymolytic* and *splenolytic* effects of COL-Ac were manifest in all groups, irrespective of whether DOC-Ac had or had not been given simultaneously. The observation that the thymus appears to be even smaller after combined treatment with COL-Ac and DOC-Ac in Group 4, than it is following administration of the former hormone alone, in Group 2, is probably meaningless. It must be kept in mind that it becomes virtually impossible to

dissect the thymus accurately for weighing if this organ becomes so atrophic that its weight falls below 30 mg. DOC-Ac given by itself did not exert any stimulating effect, either upon the indicators of inflammation or upon the lymphatic organs (Groups 3 and 6).

Discussion. The anticatabolic, pro-inflammatory, thymotrophic and splenotrophic actions of DOC-Ac are never conspicuous in intact animals, but all these effects are always very pronounced (at dose-levels used here) when DOC-Ac is given to adrenalectomized rats maintained with COL-Ac. Presumably the adrenal of the intact animal supplies an optimal amount of mineralocorticoid already so that additional DOC-Ac treatment does not produce any manifest effect(8). This is probably also the reason why the anticortisol actions of aldosterone are likewise much more evident in adrenalectomized than in intact rats(9). Hypophysectomy does not totally abolish mineralocorticoid secretion by the adrenals; hence, it might have been supposed that the inactivity of DOC-Ac in our hypophysectomized rats was likewise due to the fact that they possess an adequate supply of endogenous mineralocorticoids. However, as we have seen, DOC-Ac does not exert any evident anticortisol action, even in simultaneously hypophysectomized and adrenalectomized rats maintained on COL-Ac. It is much more probable that some pituitary factor is necessary for the manifestation of the anticortisol actions of DOC-Ac.

It had been demonstrated previously that STH does not exert any of its characteristic actions (upon somatic growth, thymicolymphatic development, inflammation) in adrenalectomized rats maintained exclusively on COL-Ac, unless mineralocorticoid treatment is given. Conversely, overdosage with DOC-Ac fails to produce nephrosclerosis, periarteritis and hypertension, in the absence of the pituitary, unless adequate maintenance therapy with STH-containing pituitary extracts, or hypophyseal implants is provided (10). It remains to be seen whether STH is the hypophyseal factor necessary for the obtention by mineralocorticoids of anticortisol actions, but the present experiments suggest to us that, not only the damaging effects of DOC-Ac upon the kidney and the cardiovascular system, but even its ability to antagonize the catabolic antiphlogistic, thymolytic and splenolytic effects of cortisol is dependent upon the hypophysis.

Summary. Hypophysectomy, with or without adrenalectomy, abolishes the anticortisol effects (upon catabolism inflammation, thymolysis and splenolysis) of desoxycorticosterone acetate, seen in the adrenalectomized rat. Apparently, the anticortisol actions of mineralocorticoids are indirect, and depend upon some hypophyseal conditioning factor.

COL-Ac ("Cortril") was generously supplied by Pfizer Laboratories, and DOC-Ac ("Cortate") by Schering Corp.

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Sodium and Potassium Concentrations in Bile from Human Gallbladders. (22873)

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Recent analytical studies on inorganic constituents of human gallbladder bile are wanting. This report is principally concerned with the concentrations of sodium and potassium in bile from human gallbladders obtained at necropsy.

Method. Analyses for sodium and potassium were made on bile after appropriate dilutions on Baird flame photometer. The pH was determined electrometrically on Beckman pH meter and total solids gravimetrically. Total pigments were determined by absorbance at 445 mμ on Beckman DU spectrophotometer and calculated as bilirubin.

Results. Specimens from 38 gallbladders obtained from 4 to 24 hours post mortem were examined. There was marked variation in volume, color and consistency of the bile. The mean sodium concentration in the 38 specimens was 169 ± 25 mEq/l with a range of 130 to 232, while the mean potassium concentration was 27 ± 11 mEq/l with a range of 12 to 68. Because of the wide variation in concentration of each of the 2 ions with no readily discernible relationship between them, additional analyses were performed on 15 of the 38 specimens. In these the mean pH was 6.72 ± 0.50 with a range of 5.79 to 7.55. The total solid content averaged 12.4 ± 4.0% with a range of 7.4 to 20.3. The total pigment concentration averaged 532 ± 462 mg % with a range of 156 to 1696 mg.

To compare the potassium and pigment concentrations in the gallbladder bile with that of hepatic bile, specimens of such bile were obtained from 5 patients with a draining tube in the common bile duct. The mean pigment concentration of bile from the common bile duct was 46 mg % and that of potassium 4.5 mEq/l.

The calculations indicated that bile from the gallbladder contained 12 times higher concentration of pigment than bile from the liver with a range of 3.4 to 38 (Table I). Furthermore, bile from the gallbladder contained 6 times higher concentration of potassium than bile from the liver with a range of 2.7 to 10.

TABLE I. Bile Concentration by Pigment and Potassium Contents Arranged in Order of Increasing Total Solids.

Total solids (%)	Concentration factors	
	Pigment	Potassium
7.4	3.4	4.5
7.6	3.8	6.0
8.5	4.2	2.7
9.4	3.4	9.8
9.5	6.2	5.8
10.5	5.6	6.2
10.8	15.9	5.0
12.0	13.6	8.0
12.8	9.1	7.5
13.1	3.5	4.7
13.5	24.8	4.1
14.2	9.2	4.0
17.6	37.9	6.7
19.2	24.9	8.4
20.3	9.2	5.1

Comment. According to the data obtained the average sodium concentration in gallbladder bile was approximately 25% higher than the average sodium concentration in extracellular fluids. The high concentrations of total solids and of total pigments may be explained by the resorption of water from the gallbladder and the lower concentrations by dilution with bile from the hepatic ducts.

The resorptive power of the canine gallbladder was estimated by Rous and McMaster(1). They observed, that measured by pigment content, the empty gallbladder concentrated bile 3.2 to 10.8 times and the full gallbladder 3.6 to 8.9 times. The resorptive power of the rabbit's gallbladder was studied by Halpert and Hanke(2). Using methylene blue, they observed that the dye reached a concentration in the gallbladder bile 2 to 22 times that in bile from the liver. Halpert, Thompson, and Marting estimated that the

rabbit's gallbladder resorbed half the volume of its content per hour(3).

Our data are in line with the above observations; namely, that certain constituents of the bile are retained in the gallbladder and reach concentrations considerably higher than that in bile from liver. Other investigators have noted a rapid loss of inorganic ions from the gallbladder(1). However, the high potassium concentrations in gallbladder bile have not previously been reported. Failure of the potassium to be resorbed and its accumulation in the gallbladder cannot be satisfactorily explained from the above determinations.

Summary. Measurements of sodium, potassium, pH, total solids and pigment in human gallbladder bile indicate wide variations. Potassium is present in higher concentrations than in extracellular fluids. The resorptive power as measured by pigment failed to correlate with the increase in potassium concentration and total solids.

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Placental and Mammary Transfer of Ingested Chlortetracycline in the Rat. (22874)

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Results with animals indicate that the maximum growth response of antibiotics occurs when they are included in the diets from earliest possible age. The oral administration of antibiotics to young animals is difficult with certain species which do not consume food in the critical period following birth. An alternate means of administering antibiotics to such animals would be through either placental or mammary transfer or both. Placental transfer of penicillin was found to occur in human subjects by Charles(1) since significant amounts were found in the liquor amnii, cord blood and maternal blood following the injection of one million units of the antibiotic during labor. Chlortetracycline, chloramphenicol, oxytetracycline and streptomycin, when given in therapeutic amounts, were shown also to be transferred in therapeutic concentrations from the maternal to the fetal tissues. Carpenter and Larson(2) observed no transfer of chlortetracycline or penicillin across the placental tissues of the sow. Chlortetracycline, however, was detected in the milk of sows following oral administration of the antibiotic but the amounts were small and variable and did not influence the growth rate of suckling pigs. Placental transfer of either antibiotic presumably failed to occur since detectable amounts were not shown in the blood serum of the pigs at birth following antibiotic administration to the sows. An earlier publication by Carpenter (3) reported that the size or viability of litters of pigs was not affected by giving chlortetracycline to gestating sows nor was the growth of the pigs influenced during the suckling period which indicated that mammary transfer did not result from the amounts consumed by the sows. Maddock *et al.*(4) were unable to obtain appreciable concentrations of chlortetracycline in the blood serum or milk from sows 8 hours after feeding the antibiotic at levels up to 20 mg per pound of ration for a week. Blood levels of 0.74 γ per ml and whey levels as high as 2.21 γ per ml were obtained, however, 8 hours after administering chlortetracycline orally in single doses at levels from 2.0 to 4.0 g. Little correlation was found between the concentration of chlortetracycline in the serum and that in the whey and milk. Uram *et al.*(5) re-

ported that young suckling rats from dams receiving antibiotic-supplemented diets gained significantly faster than controls during the first 14-day period. This indicated that placental or mammary transfer might take place in this species. The following experiments were set up to explore this possibility and to determine if detectable amounts of chlortetracycline were present in the body tissues of newborn rats from dams that had consumed diets containing elevated levels of the antibiotic.

Methods. Thirty pregnant female rats of the Wistar strain were obtained from Carworth Farms, Kingston, N. Y., approximately 2 weeks before term and randomly distributed across the 3 treatments. A total of the 10 animals were assigned to each treatment, placed in individual cages and fed *ad libitum* a standard meal diet* containing either 0, 0.5, or 2.0 g of chlortetracycline per kilo. During the afternoon of the day prior to the end of gestation the females were sacrificed. The fetuses were removed by hysterectomy, sexed, weighed, placed in identified plastic bags, quick-frozen and held until they could be assayed for chlortetracycline by a modified pad plate method. The tissues were homogenized in acid acetone (5 ml/g of tissue). Pads of filter paper, 6.35 mm in diameter, were dipped into the homogenate and placed on agar plates that had been seeded with *Bacillus cereus* (ATCC 10702). The chlortetracycline content of the samples was determined by comparing the zone of inhibition with those plotted on a standard curve, obtained by serial dilutions of a standard antibiotic solution. In the second experiment, the young were born naturally, allowed to suckle for 24 hours, sacrificed and treated as outlined above. In the third experiment, the female rats were continued on their respective treatments, rebred, and allowed normal parturition. At the end of 24 hours, the young were sacrificed and then treated as in the second experiment. The females were sacrificed, the mammary glands excised, placed into plastic bags, quick-frozen and held until assayed for chlortetracycline.

* Ingredients: Beef Meal, Animal Liver Meal, Fish Meal, Brewers' Yeast, Condensed Fish Solubles, Soybean Oil Meal, Ground Yellow Corn, Wheat Bran, Dehydrated Alfalfa Meal, Linseed Oil Meal, Oatmeal, Wheat Germ Oil, Dried Whole Whey, Soybean Oil, Riboflavin, Niacin, Calcium Pantothenate, Choline Chloride, Vitamin A Feeding Oil, Irradiated Yeast, Iodized Salt, Calcium Carbonate, Copper Sulfate, Dicalcium Phosphate, Iron Sulfate, Cobalt Carbonate, Manganese Sulfate, and Vit. B_{12} Supplement.

TABLE I. Placental Transfer of Chlortetracycline to Rat Fetuses.

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
			γ/g			
Litter No.	♂	♀	♂	♀	♂	♀
1	0	0	0	0	.40	.41
2	0	0	0	0	.28	.40
3	0	0	0	0	.37	.34
4	0	0	0	0	.33	.35
5	0	0	0	0	.34	.28
6	0	0	0	0	.42	.40
7	0	0	0	0	.41	.48
8	0	0	0	0	.16	.30
9	0	0	0	0	.41	.38
10	0	0	0	0		
Avg					.35	.37

Results. Evidence of placental transfer of chlortetracycline. Placental transfer of the antibiotic was demonstrated by detecting significant amounts of chlortetracycline in the tissues of fetuses taken one day before term from female rats that had received a diet containing 2 g of the antibiotic per kilo of diet. As shown in Table I, the antibiotic concentration average (0.35 and 0.37 γ/g) of fetal tissue showed no significant difference between males or females. It is evident that substantial amounts of the antibiotic must be

TABLE II. Chlortetracycline in Tissues of Suckling Rats.

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
			γ/g			
Litter No.	♂	♀	♂	♀	♂	♀
1	0	0	0	0	.90	.55
2	0	0	0	0	.38	.46
3	0	0	.10	.30	.37	1.00
4	0	0	.09	.08	.75	.63
5	0	0	.07	.06	.21	.34
6	0	0	0	0	.19	.21
7	0	0	.42	.39		
8	0	0	.32	.35		
9	0	0	.20	.28		
10	0	0				
Avg			.13	.16	.47	.53

incorporated into the diet of pregnant female rats to accomplish *in utero* transfer since the antibiotic was not detectable in the fetuses from rats that received the diet containing 0.5 g per kilo.

Evidence of mammary transfer of chlortetracycline. In Table II, it is shown that the tissues of young rats that suckled during a 24-hour period, contained higher levels of antibiotic than those observed for fetuses (Table I). This additional amount of antibiotic in the tissues is assumed to have come from the ingestion of the milk which indicates mammary transfer. Further evidence of mammary transfer is signified since the tissues of the young contained detectable amounts of the antibiotic after suckling dams that had received a diet containing only 0.5 g per kilo of diet.

Maddock *et al.*(4) have shown in swine that at marginal levels of antibiotic feeding, chlortetracycline will appear in the milk of some swine and not in others. This accounts for the absence of antibiotic in the suckled young of some of the dams receiving the lower level of chlortetracycline even though their stomachs contained visible milk. To verify this, all dams were rebred and their excised mammary tissue was assayed for antibiotic at the same time as their young.

Significant amounts of chlortetracycline were found in the mammary tissues of rats consuming diets containing 2 g per kilo of diet. Again the tissues of the suckled young contained antibiotic from mothers at the same dietary level (0.5 g/kg) where placental transfer did not occur and the presence of chlortetracycline in the suckled young was associated with the presence of the antibiotic in the mammary tissue (Table III).

TABLE III. Chlortetracycline in Mammary Glands and Suckling Rats.

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
	γ/g					
Litter No.	Mammary	Suckling	Mammary	Suckling	Mammary	Suckling
1	0	0	.37	1.15	.40	.90
2	0	0	.20	0	.50	1.00
3	0	0	.18	.60	.47	.46
4	0	0	0	0	0	.21
5	.15*	0	0	0	.17	.85
6	.06*	0	0	0	.18	.68
7	0	0	0	0	.80	.90
8	0	0	0	0	.58	.21
9			.58	0	des†	1.85
10					.09	.95
Avg	.02		.15	.19	.32	.80

* Probably due to natural mammary product because zone disappeared in 40 hr.

† des = destroyed.

TABLE IV. Effect of Chlortetracycline in Maternal Diet on Weight and Number of Fetuses Taken by Hysterectomy.

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
Litter No.	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g
1	10	39.0	2	10.0	11	42.0
2	10	37.0	11	39.5	10	37.5
3	10	39.0	9	32.5	11	41.0
4	4	15.5	10	39.0	11	41.0
5	5	16.0	10	38.5	12	42.5
6	2	7.5	9	35.5	9	33.0
7	9	32.5	7	23.5	12	45.0
8	10	38.5	11	41.0	8	29.5
9	10	35.0	7	38.0	9	37.0
10	10	37.0				
Avg	8	29.7	8.4	33.0	10.3	38.7

TABLE V. Effect of Chlortetracycline on Weight and Number of Young (Suckled for 24 Hr).

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
Litter No.	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g
1	9	53.0	9	49.0	8	51.0
2	8	42.0	9	51.0	11	64.0
3	9	54.0	8	43.0	8	50.0
4	6	35.0	12	63.0	11	64.0
5	8	52.0	10	54.0	10	58.0
6	9	55.0	8	46.0	4	28.0
7	8	46.0	8	46.0		
8	9	57.0	7	47.0		
9	4	26.0	8	50.0		
10	6	40.0				
Avg	7.6	46.0	8.8	49.9	8.6	52.5

Neither the numbers of fetuses nor their average weights were influenced by incorporating 0.5 or 2.0 g of chlortetracycline per kilo into the diets of pregnant rats for 2 weeks before term (Table IV). Although the average number of fetuses per dam was greater for those consuming the diet containing 2 g per kilo, the average differences were not significant. For all treatments the average fetal weights were approximately 2 g less

TABLE VI. Effect of Chlortetracycline on Weight and Number of Young (Second Litter of Dams in Table V).

Level of chlortetracycline in dams diet.

	Control		0.5 g/kg		2.0 g/kg	
Litter No.	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g	No./ litter	Wt/litter, g
1	8	52.0	11	65.0	10	62.0
2	9	60.0	11	62.0	10	59.0
3	9	60.0	8	56.0	2	19.0
4	10	63.0	12	67.0	5	37.0
5	7	36.0	10	68.0	13	82.0
6	5	32.0	3	23.0	8	44.0
7	9	63.0	14	76.0	12	77.0
8	5	33.0	4	32.0	7	50.0
9			13	84.0	13	80.0
10					3	20.0
Avg	7.7	49.9	9.5	59.2	8.3	53.0

than for the young rats that were sacrificed at 24 hours after birth.

The high level of chlortetracycline supplement appeared to aid in conception on second breeding. In the second experiment (Table V), 4 of the 10 females received did not bear young. When these same females were continued on the same supplement while being rebred, all conceived within a 3-week period and went full term (Table VI). In the unsupplemented group conception was slower and only 8 of the 10 females conceived over a period of 3 months.

Summary. Pregnant female rats were fed diets containing different amounts of chlortetracycline. When the prenatal diet contained 2 g/kg the antibiotic was present in the fetal tissues. At levels of 0.5 and 2.0 g/kg of diet, detectable amounts of the antibiotic were present in the mammary glands of the females and in tissues of the suckled young. The higher level of antibiotic (2 g/kg) improved the conception rate but did not affect the size of the fetuses or the number of young per litter.

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Radioactive Tracer Studies on Uptake of Diamino-diphenyl-sulphone by Leprosy Patients. (22875)

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Diamino-diphenyl-sulphone and a number of its derivatives are now well-established in the treatment of leprosy. But there is much confusion as regards the mode of action of sulphone on leprous lesions(1-5). The present radioactive tracer study on uptake of sulphone by leprosy patients was undertaken with the hope that it might throw some new light and may be helpful in removing the confusion. The investigation has been concerned with (a) concentration of the drug in blood, bone-marrow and skin tissues, the rate of excretion through kidneys and (b) the localization, if any, of the drug in affected tissues and invading micro-organisms. This paper deals with the first part of the investigation.

Material and methods. We studied 22 cases of leprosy—8 lepromatous and 14 non-lepromatous. They were adult males, weighing 40-60 kg. Blood volume of each patient was determined by Evans blue and thiocyanate technics. *Diamino-diphenyl-sulphone (DDS)* tagged with S-35 was used. This was procured from Amersham, England and had an initial specific activity of 1 μc/mg. For *oral administration,* a single dose of 4 μc/mg was given as a powder. For parenteral use, 2-7 μc were injected subcutaneously as a 5% suspension in 0.1% agar solution. *Blood* was

taken at 1, 2, 4, 6, 10, 24 hours after administration and then every 24 hours for several days until the concentration of the drug could not be recorded. *Urine* of each patient during 24 hours was pooled to study the cumulative renal excretion for 10 consecutive days. Urine passed at different times within the first 24 hours was also collected for study of rate of excretion. *Bone marrow* was obtained by usual sternum puncture technic. *Skin tissues* (both healthy and affected) were obtained by biopsy under local anesthesia employing nerve blocking technic. The tissues were finely minced and homogenized with distilled water in a specially devised homogenizer. Blood, urine, bone-marrow or suspensions of skin tissues were then uniformly spread over previously-weighed copper planchets of 5 cm^2 area and dried under an infra-red lamp. The planchets were then reweighed. Drying and weighing was repeated till final weight became constant. The difference of final and initial weights divided by area of planchets gave the thickness of dried samples in mg/cm^2. Thicknesses varied from 1-5 mg/cm^2. Radioactive assay: Each planchet was placed in a fixed position under an end-window G-M counter (window thickness 2.5 mg/cm^2) surrounded by 1″ of lead. The number of counts/min. n, due to beta particles of S-35 of radioactive DDS in the sample was then determined. The corresponding activity, a, in μc, could be deduced from the following formula:

$$a = [(1.44t)/(1 - e^{-0.225t})] \times n \times 10^{-6} \quad (1)$$

Where t is thickness of sample in mg/cm^2. This relation had been derived from previous calibration experiments.

Observations. Concentration of DDS in blood. From the G-M counter data the concentration of DDS/ml of blood could be obtained from the equation (1). Total concentration, *i.e.*, amount of DDS in total blood was determined by multiplying this figure by blood volume of patient. The latter varied from 3,250 to 5,400 ml, average 4,333 ml. Variation of average total concentration of patients having a single oral dose is shown in Fig. 1. The vertical bars indicate standard

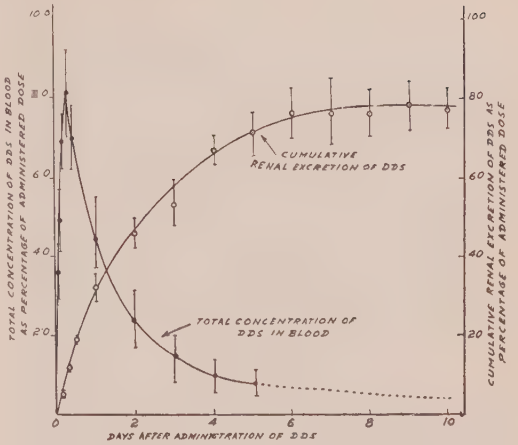

FIG. 1. Total concentration of DDS in blood and its cumulative renal excretion expressed as % of administered dose.

deviations of average values. The concentration increased very rapidly during initial hours and reached maximum level of 8.1 ± 1.1% of administered dose at about 6 hours after administration. Then it began to decrease somewhat slowly to 4.6 ± 0.9% at the end of 24 hours. After 4 days, it reached a negligibly low level of 0.96 ± 0.4%. At the end of 5 days the amount of tagged DDS in blood was too low to be detected by our method.

Renal excretion. Since it is known from the chemical estimation(6) that most of the sulphone is excreted through the kidneys, only renal excretion was studied in this experiment. The variation of average cumulative excretion after a single oral administration is presented in Fig. 1. As before, the bars indicate standard deviation of the average. As shown in this Figure most of the sulphone (about 76%) had been excreted within 6 days, and a slight increment, ~ 2% in the next 4 days.

Concentration in bone marrow. Bone marrow samples were collected from 6 patients, at 24, 72, 120 hours respectively. The level of activity was roughly that of blood at corresponding periods.

Concentration in skin. Uptake of the drug by skin tissues was investigated to elicit whether there was preferential fixation of DDS and if there was any significant difference in concentration of the drug in healthy

TABLE I. Concentration of Radioactive Diamino-Diphenyl-Sulphone in Blood and Skin Tissues.

Dose	Hr after admin.	Conc. (in μc/g) Blood	Healthy skin	Affected skin	Nature of lesion
Oral admin.:					
4 μc/kg	6	.035	.013	.143	Single
body wt	6	.036	.016	.067	Multiple
	18	.019	.014	.140	Single
	18	.008	.005	.064	Multiple
	24	.008	.009	.084	Single
	24	.010	.005	.083	Multiple
	24	.019	×	.050	Generalized
	72	.001	0	.004	Multiple
	144	0	0	.010	Single
	144	0	0	.005	Multiple
Subcut. inj.:					
2 μc	3	.001	.001	.012	Single
5 "	6	.002	.003	.016	Multiple
7 "	48	.001	.002	.050	Single

and affected tissues. Patients suffering from single and multiple lesions were studied. The findings in Table I show that the concentration in healthy skin during the first 6 hours was about 2 to 3 times less than that of blood. The difference was gradually made up and at the end of 18 hours the concentration of DDS in blood and healthy skin tissue was about the same. However, uptake of the drug by the affected tissues was always about 10 times greater than that of healthy tissues. No significant difference in concentration was observed in single versus multiple lesions.

The much larger localization of DDS in affected skin tissues after oral administration suggested a study of the same localization after subcutaneous injection of the drug. The injection was given at a site adjacent to the affected zone but about 4" away from it. It was found that the drug was, as before, preferentially localized in the affected tissues.

Discussion. It has been noted earlier that diamino-diphenylsulphone when administered by mouth could be detected in blood within a short time after its ingestion. Concentration of the drug in blood reached its maximum in about 6 hours, but the total quantity of the drug in blood constituted only about 8% of the total dose. The corresponding concentration in blood was only about 0.6 mg%. As the solubility of the drug in water is 10 mg% and had the drug passed directly from stomach to blood stream, the maximum concentration in blood should have been much larger as well as quicker. It therefore seems probable that the drug is first localized in the cells of gastrointestinal tract from where it is slowly taken up by the blood.

When the daily rate of diminution in blood is compared with the rate of excretion through kidneys during this period, another interesting feature of sulphone uptake becomes apparent. These rates could be determined in the following way: When the average total concentrations in blood (Fig. 1) were plotted against time in a semilog graph, it was found that, barring initial interval of 10-12 hours, the points approximately fell on a line of slope 0.62 per day. This showed that the concentration in blood diminished more or less exponentially with time, the daily rate of diminution being 62%. To obtain renal excretion rate, a hypothetical kidney system had to be visualized, from which the drug was gradually excreted. The initial concentration in this hypothetical kidney system should be taken as equal to total amount of drug actually excreted through the kidneys *i.e.* about 78% (Fig. 1). By deducting the cumulative total excretion from this value (*i.e.* 78%), the percentages of the drug retained at any instant in this system could be obtained. On plotting these percentages on semilog paper against time, an approximate straight line curve of slope 0.42/day was obtained. This meant that the drug cleared out of the kidneys almost exponentially, the rate of clearance being about 42%/day.

Up to about 18 hours after administration, concentration in blood remains higher than that of skin, nerve, and bone marrow but after that period, concentration stabilizes and the drug was more or less in the same concentration in blood, bone marrow, healthy skin tissues. However, that the concentration in leprous tissues was about 10 times more than in healthy ones, explains, at least partially, the fate of the extra quantity of the drug which disappears from blood, but could not be detected in urine. As regards mode of localization of the drug in the affected tis-

sues it might be mentioned that Chatterjee *et al.*(7) had shown that the *M. leprae* and leprous tissue contained mucopolysaccharides. Leprous tissue and bacilli containing mucopolysaccharides might have some special affinity for DDS.

Of the total quantity of drug administered, about 78% could be accounted for in the urine. It may be argued that a certain percentage was excreted through other channels and a portion might have been lost in manipulation and the balance localized in sensitive sites. As recorded above, the concentration in blood and urine was too low to be detected by our method 10 days after administration. Is it possible that the drug was localized in a sensitive zone and was excreted at a very slow and reduced rate? Cornbleet(8) while studying the pharmacology of sulpha drugs found that after a sweat bath the drug could be detected in blood, skin, sweat and urine several days after its disappearance. In at least one of our cases a skin biopsy from the affected site, 14 days after administration of the drug showed presence of DDS, though concentration in blood and urine was not detectable several days before date of biopsy. It may, therefore, be concluded that the drug was localized in affected tissues from where it is slowly excreted through excretory organs.

Summary. 1. The uptake of sulphone by leprosy patients was investigated, employing S-35 tagged diamino - diphenyl - sulphone (DDS). After oral administration: (i) total amount of drug in blood increased rapidly during initial hours reaching a maximum of about 8% of dose at 6 hours and then gradually decreased at an average rate of 62% per day to a negligible level in about 5 days; (ii) on an average 78% of the drug was excreted through the kidneys within about a week; the average rate of excretion being 42% per day; (iii) level of drug in bone-marrow and healthy skin was almost parallel to that in blood; (iv) the drug was preferentially localized in diseased skin tissues, where concentration was about 10 times greater than that in healthy tissues. 2. Subcutaneous injection also revealed similar preferential localization in affected regions. The significance of these findings is discussed.

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Microdetermination of Acetylable Steroids in Plasma.* (22876)

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Many of the steroid analytical methods described in the literature have contributed to the present knowledge of steroid metabolism; however, they have been limited in application due to relatively low sensitivity and specificity. Methods based upon chromogenic procedures(1-3) are generally specific for molecular groupings on the steroid molecule, not individual steroids. Polarographic(4) and fluorometric(5) procedures are relatively specific, but are applicable to only a limited number of steroids. Paper chromatography procedures(6,7), although relatively specific for a wide variety of steroids, have presented problems in quantitation.

The method described here employs a combination paper chromatography-radioactive isotope technic which gives high sensitivity and specificity in an analytical range not heretofore possible. In this method, steroids with 3, 20, or 21-hydroxyl groups (includes all corticosteroids) are acetylated with radioactive acetic anhydride-1-C^{14} (S.A. 2 millicuries/millimol)† in pyridine. After chromatography, the radioactivity of the steroid acetates is determined directly from the paper chromatogram. C^{14} acetylation for identification of steroids has previously been employed(8), but neither details of the procedure nor the quantity of steroid acetylated has been reported. Five to 10 ml of plasma are extracted with chloroform, the chloroform evaporated *in vacuo*, and the fatty residue partitioned between hexane and 75% methanol. The hexane is discarded and the methanol evaporated and re-extracted with chloroform. The extract is dried and acetylated with 700 μg of radioactive acetic anhydride in pyridine overnight at room temperature. This fraction contains the "free" steroids. A beta-glucuronidase hydrolysis(9) of the plasma residue is carried out and the hydrolysate extracted with chloroform, evaporated and acetylated in the above manner. This fraction contains the steroid glucuronides. After acetylation is completed, the excess acetic anhydride is removed by a small stream of nitrogen. Two sodium hydroxide traps are employed in a train to catch the excess radioactive acetic anhydride. The dried residue is dissolved in a small quantity of chloroform-methanol 1:1 and chromatographed on paper according to the method of Zaffaroni(6). After chromatography, the paper is dried in air and passed through an automatic strip counter. The radioactive area on the recording paper is measured by a planimeter to determine the quantity of steroid. To determine the quantity of acetic anhydride required to react quantitatively with a known quantity of steroid added to the plasma, radioactive 4-C^{14} labeled corticosterone and tetrahydrocortisone in quantities of 1 and 1.4 μg respectively were added to 5 ml sample of plasma and carried through the above procedure. The samples were acetylated with various quantities of non-radioactive acetic anhydride. Fig. 1 illustrates data which show quantitative recovery when quantities of acetic anhydride greater than 500 μg were employed. The chromatographic system employed varies with the steroid to be determined. The isolation and quantitation of a single hormone—cortisol—will be taken as an example. After the plasma has been extracted with chloroform‡, and the extract dried and acetylated, 100 μg

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† This can be diluted in accordance with the quantity of steroid acetlyated.

‡ An alternate procedure for extraction of cortisol from plasma has proven advantageous. The plasma is first extracted with heptane to remove fat and other low polar substances. A subsequent extraction with chloroform yields a quantitative recovery of cortisol essentially free from other interfering compounds. Steroids more polar than cortisol (Tetrahydrocortisol) may be extracted quantitatively by substituting benzene for heptane and subsequently extracting with chloroform.

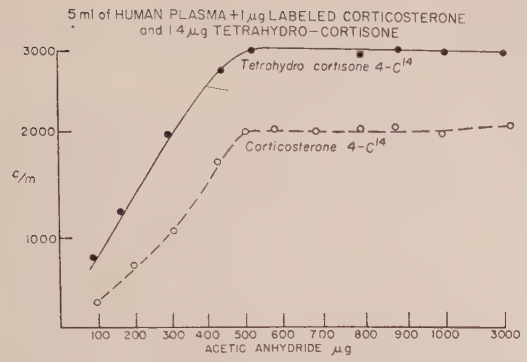

FIG. 1. Quantitative recovery of radioactive corticosterone and tetrahydro-cortisone with various amounts of non-radioactive acetic anhydride.

of non-radioactive carrier cortisol acetate are added. The mixture is then chromatographed in the benzene-formamide system for 5 to 8 hours. After the paper is dried, the paper chromatogram is analyzed for radioactivity by means of a recording strip-flow counter; the position of the added cortisol is detected with a Haines-type scanner(10). The cortisol area is then eluted with methanol and the specific activity of the eluent determined as follows: An aliquot of the eluent is analyzed spectrophotometrically to determine the total quantity of alpha-beta unsaturated ketone present. The quantity of steroid in the plasma as represented by the radioactivity is then determined by the following equation:

$$\frac{F \times A \times M}{B \times C} = \mu g \text{ steroid in plasma sample.}$$

F = the beta ray self absorption factor of the paper. This factor is very constant for any particular system and paper, but must be determined for each system.§ In our present system, this is 6.12.

A = radioactivity area in c/m

M = molecular weight of steroid in μg.

B = fraction of added steroid recovered. In our system this has been 0.7-0.9

C = specific activity of acetyl radical in c/m/μmole. This must be determined for each counting system.

Results. When known quantities of cortisol are acetylated with radioactive acetic anhydride, a linear curve between corrected counts per minute and μg cortisol is obtained; this allows direct reading from a graph.

A simple procedure is employed to confirm the identity of an unknown compound. After the specific activity is determined as above, the mixture is oxidized with CrO_3(6), and rechromatographed in the same system. If the substances are identical they migrate at the same rate on the paper and the specific activity is constant(11). This technic is illustrated in Fig. 2. The specific activities were found to be identical in both the untreated and oxidized steroid.

Six samples of plasma were obtained at 3:00 p. m. from normal subjects and analyzed for cortisol content. The mean value determined corresponds to 0.86 microgram per 10 ml of plasma with a standard deviation of 0.042. 410 ml of pooled normal plasma were carried through the same procedure, using an excess of radioactive acetic anhydride. From this plasma 36.2 μg of cortisol acetate were isolated and identified by both radioactive (11) and chemical(6) methods. This level corresponds to 0.88 μg/10 ml of plasma, which is comparable with those obtained by the micro-method technic.

Other compounds have been isolated and identified by the same procedure: cortisone from the free fraction, and tetrahydrocortisol, tetrahydrocortisone and pregnandiol from the glucuronide fraction. Additional identifications are in progress. Quantities of less than 0.1 μg steroid have been detected by this method. The sensitivity depends on the specific activity of the labeled acetic anhydride used. Sensitivity is greater for steroids having more than one acetylable hydroxyl group. The application of this method to studies of fluids and tissues in diseased states will be reported elsewhere.

Summary. A method is described which employs a combination of paper chromatographic and radioactive isotope technics. It is applicable to a wide number of hydroxylated steroids and is specific and sensitive to less than 0.1 μg quantities of steroids. The sensitivity is dependent upon the number of hydroxyl groups and specific activity of the radioactive acetic anhydride which is used to

§ To be published.

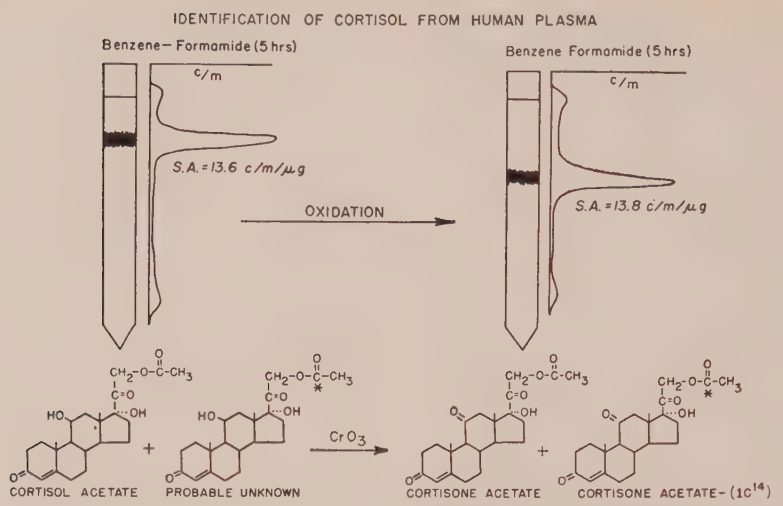

FIG. 2. Chromatographic procedure for identification of cortisol. Specific activity (S.A.) of cortisol acetate after oxidation remains constant.

label the steroid. It has been applied successfully to the analysis of steroids in blood, urine, and a variety of tissues.

The author wishes to acknowledge the helpful advice and friendly criticisms of Dr. Thomas F. Dougherty, Dr. Leo T. Samuels, and Dr. Max L. Sweat.

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Release of Potassium from the X-Irradiated Mammalian Heart.* (22877)

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The observations of a number of investigators indicate that a loss of potassium from the cell may be a general effect of exposure to ionizing radiation. Adler and Wiederhold(1) observed an increase of potassium in the urine of rabbits for the first 4 or 5 days after exposure to X-ray. Edelmann noted similar urine changes in rats exposed to as little as 500 r. Moon *et al.*(2) found an increase in blood potassium levels in X-irradiated dogs. Bowers and Scott(3) observed an increase in urinary and fecal excretion of potassium, and a loss of potassium from radiosensitive tissues and from bone 1 to 3 days after X-irradiation of rats. Sheppard and Beyl(4) demonstrated that erythrocytes lose potassium and gain an equivalent amount of sodium when exposed to X-rays *in vitro*. On the other hand, there was no decrease in the potassium content of the muscle of the rat forelimb when examined 22 hours after local X-irradiation with doses up to 73,000 r(5). In the above studies the first analyses for potassium were made on blood samples or tissues taken about one day after irradiation of the animal. Goodman and Vogel(6), however, measured the serum potassium in rabbits within the first two hours after exposure to 850 r, but found no consistent changes.

For the present study the mammalian heart was selected as a suitable test object for the investigation of the effect of X-ray in causing a disturbance of potassium distribution in a functioning organ. Zwaardemacher(7) postulated but did not demonstrate that potassium is released from the irradiated myocardial cell of the isolated frog heart.

Methods. The hearts from adult albino rabbits were perfused with Locke-Ringer solution by the Langendorff method at a constant temperature of 38°C and under a constant head of pressure. Perfusate samples for potassium analysis were collected in 100 ml beakers set in circular depressions on the periphery of a turntable placed beneath the heart. This table could be rotated by remote electrical control, and a series of 25 samples of perfusate could be collected at any desired sampling interval during the irradiation of the heart. To obtain coronary sinus blood from hearts irradiated *in situ* dogs were anesthetized with 150 mg/kg Na-phenobarbital injected intraperitoneally. Blood pressure was recorded from a femoral artery with a mercury manometer. The sternum was removed and artificial respiration was applied through a tracheal cannula. The pericardium was cut down the midline and arranged as a sling to lift the heart away from the moving lungs. To facilitate the drawing of venous blood from the coronary circulation the apex of the heart was lifted up by hand to expose the coronary sinus at the A-V junction. Ten ml blood samples were drawn from the sinus through a 20 gauge needle. Two femoral vein blood samples and two coronary sinus blood samples were drawn during a 45-minute control period before the heart was irradiated. The clotted blood was centrifuged and the serum removed. Each serum sample was tested for extent of hemolysis by a determination of its hemoglobin content. For this purpose 1 ml was alkalinized with NH_4OH, diluted to 10 ml with distilled water and read at 541 Å in the Beckman Spectrophotometer.

Potassium analyses. A Perkin-Elmer model 52-A flame photometer was used for K analyses. The K in each lot of Locke-Ringer solution was estimated by the direct method in which the unknown was read in terms of per

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cent of the K content of a standard Locke-Ringer solution. Each sample of perfusate was tested alternately with a Locke-Ringer control in order to take into account any change in sensitivity of the instrument during the analyses. One ml of the coronary sinus blood serum from the *in situ* dog heart was diluted with 23 ml of distilled water and 2 ml of a 2% lithium chloride solution and analyzed in the flame photometer for Na and K. The internal standard method was used and a standard curve was prepared for each set of samples before and after analysis of the unknown samples of serum. The average of the 2 curves was used to calculate the amounts of Na and K in the serum.

Irradiation. A Keleket Therapy X-ray machine was used, operated at 250 kilovolts and 15 milliamperes. The amount and rate of X-ray delivered was determined by Victoreen thimbles and a constant recording integron. No filter was used except that inherent in the machine (equivalent to 0.25 mm of Cu). All isolated rabbit hearts were placed 25 cm from the target and were irradiated at the rate of 500 r per minute. A therapy cone with a 4-inch opening was used in the irradiation of the *in situ* dog hearts (60 cm target distance, 150 r per minute) to shield the rest of the animal and allow exposure of only the heart and the tissues beneath it. Control procedures carried out either before the beginning of irradiation or with non-irradiated hearts are outlined in appropriate sections in the description of the results.

Results. Potassium release from perfused rabbit hearts. Following a 30-minute control period the irradiation of the rabbit hearts was started and continued for 2 hours and 25 minutes. Samples of perfusate were collected at 10-minute intervals during the control period, at 1 minute intervals during the first 10 minutes of irradiation, at 5-minute intervals for the next 15, and at about 15-minute intervals for the next 2 hours. The rate of perfusion remained constant throughout this period, and the rate and amplitude of beat remained unaffected by the exposure, showing only the minor fluctuations seen in unirradiated hearts run under the same conditions.

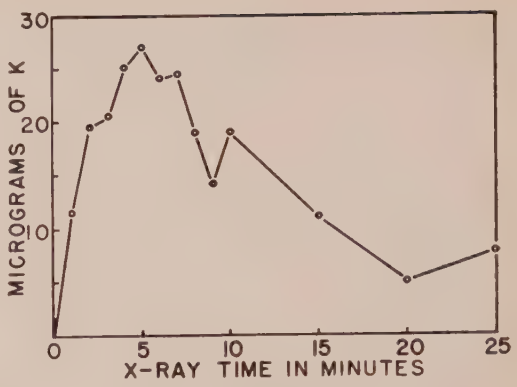

FIG. 1. Increase of potassium/ml of Locke-Ringer perfusate during continuous X-irradiation (500 r/min.) of isolated rabbit hearts. Each point represents the mean of results from 10 hearts.

The results of the K analyses of the perfusate fractions collected during the first 25 minutes of exposure are summarized in Fig. 1. In all experiments the pre-irradiation control values, averaging 225 μg of K per ml, remained constant at the level represented by zero on the graph. After the 25-minute exposure period, the K levels fluctuated between 5 and 15 μg/ml above the control value during the 2 hours of continued radiation.

Since the K concentration in the perfusate appeared to increase in at least 1 minute after the start of irradiation the effect of small doses of X-ray was studied. Seven isolated hearts were irradiated with doses ranging from 100 to 500 r (Table I). The lowest dosage at which a definite increase of K appeared in the perfusate was 500 r. A questionable increase resulted from exposure to 450 r. Hearts exposed to 400 r or less exhibited no evidence of change in their perfusate K con-

TABLE I. Increase of Potassium in Perfusate of Isolated Rabbit Hearts after Short Exposure to X-Ray (1 Heart at Each Dose Delivered at 500 r/Min.).

X-ray dose (r)	Maximum % K increase	Time of maximum increase, after start of X-ray (min.)
100	.0	
250	.0	
400	.0	
450	5.7	4
450	3.0	5
500	5.6	9
500	12.0	8

TABLE II. Potassium Lost in Perfusate of Irradiated and Non-Irradiated Rabbit Hearts. (6 in each series.)

Total perfusion time in min.	Dose of X-ray (r)	K recovered in perfusate over that in perfusing medium† (mEq/kg of heart)
90*	1000	27.33 (13.7 -45.1)
90	None	6.23 (2.65-12.7)

* Irradiation started after 30 min. perfusion.
† K measured in perfusate collected during last 60 min. of perfusion.

centrations.

To obtain an estimate of the amount of K lost by the heart as a result of a given dose of X-ray 12 rabbit hearts were perfused for 90 minutes, 6 of them being exposed to 1000 r after a control period of 30 minutes. The perfusate of each heart was collected during the last 60 minutes of perfusion and its K content measured. The results of this experiment are summarized in Table II, where it is seen that on the basis of heart weights the irradiated hearts lost more than 4 times as much K to the perfusate in 1 hr as did the control hearts.

Potassium release from in situ dog heart. The changes in the K content of the serum from the coronary sinus blood of irradiated and non-irradiated dog hearts are represented in Fig. 2. More striking than the increase in the K levels in the coronary sinus serum of the irradiated hearts is the difference between the latter change and that seen in the control tests. The decrease in the serum K in the control hearts may have been a reflection of a decrease of K in the general circulation attributable to the operative procedures. Two animals, anesthetized but not operated upon, did not show a decrease in the K of the femoral venous serum, whereas 2 dogs operated upon and not irradiated showed 10% and 28% reductions in femoral serum K at comparable time intervals of one hour after the start of the experiment. It is seen that in all irradiated hearts the K levels reached a maximum 20 minutes after irradiation was started, and then in each case began to diminish, in spite of the fact that the times required to deliver the X-ray doses varied from approximately 3 to 80 minutes.

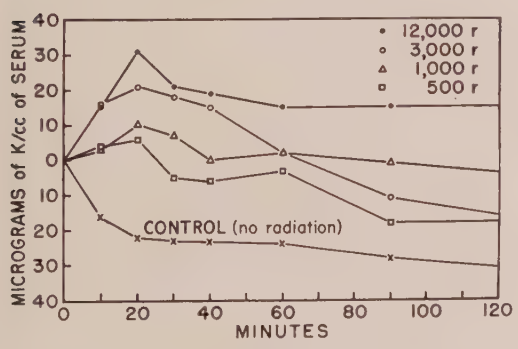

FIG. 2. Change in potassium conc. in blood serum from the coronary sinus of dog hearts irradiated *in situ* at 150 r/min. Time is measured from beginning of irradiation. Points represent mean values of 2 control animals, 2 exposed to 500 r, 4 to 1000 r, 2 to 3000 r and 1 to 12,000 r.

The Na levels in the coronary sinus serum, measured in each of the samples in which K was measured, showed no evidence of significant change under the influence of X-irradiation (Table III).

TABLE III. Serum Sodium (mEq/l) in Coronary Sinus Blood of X-Rayed Dog Hearts.

Time from start of irrad. (min.)	No. of dogs at each X-ray dose: 1 12,000 r	2 3,000 r	4 1,000 r	2 500 r	2 Control
0	146	146	153	161	151
10	141	"	154	163	150
20	146	147	"	161	"
30	"	145	151	163	"
40	"	146	153	161	151
60	"	143	155	164	"
90	148	140	"	159	"
120	146	145	153	166	"

Hemoglobin readings were made on each sample of serum in order to determine the degree of hemolysis and the possible release of K into the serum from the erythrocytes. The degree of hemolysis of the blood was found to have no significant effect on the serum K levels. The red cells of the dog contain only 4 to 11 mEq of K per liter(8), or at most only twice as much as does the serum. The greatest degree of hemolysis seen in our samples accounted for only 0.32 μg of K per ml of serum. For this reason the dog is a convenient animal to use in this study in contrast to some other species in which small degree of hemolysis could cause a relatively large K release in the serum.

The blood pressure of the dogs, recorded for 2 hrs from the start of the irradiation of the hearts, decreased gradually throughout this period. The greatest decline was 30 mm Hg, seen in the dog receiving 12,000 r; only a slight decline was seen in the 2 non-irradiated controls. Though little significance can be attached to these observations in so few dogs it is evident that the change in K concentration in the coronary sinus blood was not secondary to alterations in blood pressure. The K levels changed before a significant fall in blood pressure was seen.

Discussion. The results show that the perfused isolated rabbit heart and the *in situ* dog heart begin to lose K almost immediately when exposed to moderate doses of X-ray. When the heart was irradiated continuously the maximum rate of K release occurred after 2,500 to 3,000 r had been delivered to either the rabbit heart or the dog heart, though the former was irradiated at 500 r/min and the latter at 150 r/min. Thus the rate of loss of K from the isolated rabbit heart was greatest after 5 minutes of irradiation, as compared to 20 minutes for the *in situ* dog heart, at which times both preparations had received approximately equal doses of X-ray.

A point of interest is the rapidity of the onset of the effect observed and the relatively low dose of irradiation which caused it. An obvious question is whether irradiation of other perfused organs would result in the same phenomenon. The studies of others(1, 2,3) reveal an influence of similar X-ray doses on K blood levels and excretion in intact animals but they do not reveal such an immediate effect. This involves no real discrepancy, however, considering the widely different experimental conditions used. But in the experiments of Goodman and Vogel(6) one might have expected to see an increase in the K level of the rabbit serum drawn during the first hour after irradiation of the whole animal, if other organs participate in this early K release. Whether this immediate liberation of K from the heart in response to radiation is a phenomenon specific to that organ is unknown. If such were the case, the liberation of K from only the heart would be impossible to detect in the systemic blood of the whole animal. In this connection it would be of interest to test other perfused organs, perhaps a "radiosensitive" one such as the spleen, in the manner in which the heart was tested here.

The mechanism by which X-rays cause the release of K from the heart is not known. An interference with the controlling mechanism in the cell membrane which regulates its permeability and the balance between the intra- and extracellular constitutents, may be a factor concerned in the effect. Contraction is accompanied by a loss of K from the heart muscle fiber. Since energy is required for its replacement it is conceivable that ionizing radiation impedes the return of K to the cell by interfering with energy producing mechanisms. By this reasoning, radiation would be expected to have less effect on resting skeletal muscle, in which K movement is less rapid. The present findings in the dog heart neither reflect nor refute the possibility of an equivalent K-Na exchange in the irradiated heart cell such as has been observed in the irradiated erythrocyte(4). Even if this phenomenon occurs in the irradiated dog heart cell it would be difficult to detect by measuring the K and Na content of the coronary sinus serum, since an increase of 0.5 mEq/l of K, which constitutes about a 12% change, would be accompanied by less than a 0.5% decrease in Na, which would be impossible to measure under the experimental conditions employed.

Summary. 1. X-irradiation caused a release of potassium from the isolated rabbit heart, the rate of release reaching a peak after a dose of about 2,500 r delivered at 500 r/minute. The greatest average increase of potassium in the perfusate was 12% over the control level. 2. Potassium is liberated from the isolated rabbit heart in small amounts following exposure to as little as 500 r of X-ray. 3. Isolated rabbit hearts exposed to 1000 r released into their perfusate more than 4 times as much potassium in 1 hr as did non-irradiated hearts. 4. The X-irradiated *in situ* dog heart also released potassium into the coronary circulation. The magnitude of the effect and the radiation dose producing it

were approximately the same as in the isolated rabbit heart.

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Effect of STH on Experimental Lathyrism.* (22878)

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The literature on lathyrism and our earlier observations on the influence of hormones upon this condition had been reviewed elsewhere(1,2). Therefore, it will suffice here, to summarize a few facts which are particularly pertinent to the subject of this communication. It has been possible to produce skeletal deformities and aortal aneurysms, similar to those obtained by feeding sweet-pea (*Lathyrus odoratus*) seeds, by the subcutaneous administration of aminoacetonitrile (AAN) to rats. In very young AAN-treated rats aortal aneurysms are common, but in animals weighing 100 g or more, the drug tends to produce widespread skeletal deformities without any manifest lesions in the aorta. In these older rats, treatment with cortisol can suppress the skeletal lesions without sensitizing for the production of aortal aneurysms. Conversely, treatment with desoxycorticosterone exerts no marked effect upon skeletal lathyrism but sensitizes the rat to the production of aortal aneurysms. Finally, combined treatment with desoxycorticosterone and cortisol can completely reverse the normal morphologic syndrome of experimental lathyrism in that this corticoid combination causes AAN to produce aortal aneurysms in the virtual absence of bone lesions.

* These experiments were supported by Grants from the Ministry of Health of the Province of Quebec, Canada, and the National Research Council of Canada.

The object of this communication is to describe the effect of somatotrophic hormone (STH) upon intoxication with AAN. Our earlier experiments had shown that in general there is an antagonistic interaction between STH and glucocorticoids in the regulation of tissue responses to various types of injury (3).

Materials and technics. Two experiments were performed to explore the effect of STH upon acute and chronic AAN overdosage respectively. In the first experiment 20 female Sprague-Dawley rats, having a mean initial body-weight of 55 g (range 48-62 g), were subdivided into two equal groups. All animals received 5 mg of AAN (Aminoacetonitrile hydrosulfate) and 1 mg of STH (Armour), both compounds being given in 0.2 ml of water, twice daily, subcutaneously. Throughout the period of observation the rats were maintained exclusively on "Purina Fox Chow" and tap water. Towards the end of the first week all the STH-treated animals were obviously sick and developed the wobbling walk characteristic of experimental lathyrism, while the controls showed only a very slight impediment of locomotion. By the tenth day 5 of the STH-treated animals were manifestly moribund; these were killed, together with one rat of Group I, that was to serve as a control. At the same time AAN injections were stopped, while STH treatment continued at the same dose level for an ad-

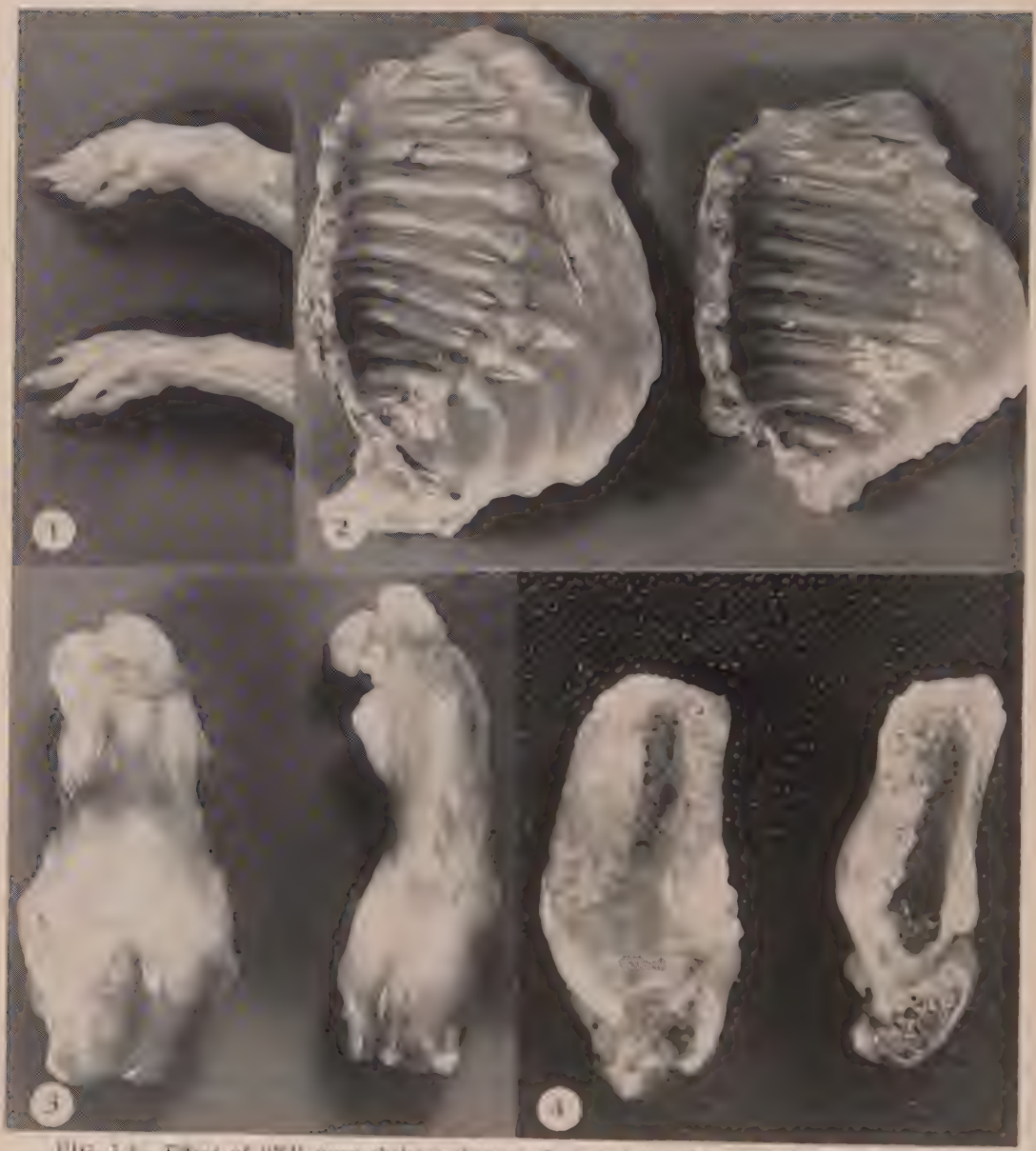

FIG. 1. Excessive bone and cartilage proliferation in small wrist joints of rat treated with STH and AAN (*top*) in comparison with virtually normal appearance of such in control animal treated with AAN alone.

FIG. 2. Considerable swelling at costochondral junctions, with periosteal bone proliferation along entire length of ribs, in rat treated with AAN + STH (*left*), compared with much milder changes in control rat treated with AAN alone.

FIG. 3. Monstrous deformation of femur with excessive periosteal bone proliferation, numerous large exostoses at muscle insertion sites, and intense proliferation of cartilage in distal epiphyseal plate, after treatment with STH + AAN compared with milder but still marked deformations in femur in control rat that received AAN only.

FIG. 4. Cross-section through femurs shown in Fig. 3. Here intense proliferation of bone, complete disruption of normal epiphyseal plate structure and marked overgrowth of cartilage are even more evident.

ditional 10 days; the remaining rats of both groups were then killed.

The second experiment—designed to explore the effects of STH upon a more chronic AAN intoxication—was performed on 30 female Sprague-Dawley rats weighing 93-105 g (average 101 g). The animals were subdivided into 3 equal groups; Group I acted as untreated controls, Group II received only AAN and Group III, AAN + STH. STH (Armour) was administered, as in the previous experiment, at the dose of 1 mg in 0.2 ml of water subcutaneously twice daily throughout the experiment. AAN was given, first at the comparatively low dose level of 3 mg once a day and then raised, on the fourteenth day, to 5 mg twice a day. At both dose levels the drug was dissolved in 0.2 ml of water and injected subcutaneously. By the thirty-first day 2 of the animals in the group receiving AAN + STH had died, and showed extraordinarily marked skeletal deformities, while the others were obviously in poor condition. Therefore, AAN treatment was stopped for a period of 5 days to permit recovery of the surviving animals, but STH administration was not discontinued. After this interval AAN treatment was resumed at a dose of 5 mg twice a day and continued until the termination of the experiment on the fortieth day.

Results. In the *first experiment* the rats killed on the tenth day showed pronounced signs of acute AAN overdosage. There were marked bone deformities and hemorrhages at tendon insertion sites and along the ribs in every rat, but the changes were much more pronounced in animals receiving STH + AAN than in the controls treated with AAN alone. The blood coagulation time was not determined but it was obvious at autopsy that clot formation was greatly delayed.

Immediately after discontinuation of the AAN injections, the surviving animals showed obvious signs of recovery, but on the tenth day, when these rats were killed, the skeletal deformities were still very pronounced though much less so in Group I, which received AAN alone, than in Group II which was simultaneously treated with STH. Proliferation of bone tissue at muscle insertion sites, and excessive growth of both bone and cartilage at epiphyseal junctions, were obvious in the wrists (Fig. 1), the costochondral junctions of ribs (Fig. 2), the femur (Fig. 3) and in virtually all other parts of the skeleton. The histologic structure of these lesions was quite typical of AAN intoxication, as characterized in our earlier publications(1,2), and hence need not detain us here. It is noteworthy, however, that under these conditions STH did not stimulate anabolism; by the end of the experiment the mean bodyweight gain of the STH-treated animals was 20 g, that of the controls 22 g. Further, the dissected femurs were actually shorter in the former than in the latter group (Fig. 8).

Although STH greatly aggravated the morphologic changes and increased the mortality due to this acute overdosage with excessive amounts of AAN, it was thought advisable to repeat the experiment on larger, and hence more resistant, rats, starting with smaller doses of AAN, so as to bring out the effect of STH more clearly. This was accomplished in the *second experiment*. Here, AAN alone produced barely detectable skeletal lesions (without hemorrhages or mortality) while in combination with STH it caused considerable systemic damage and two deaths; the controls were still in excellent condition on the fortieth day when the experiment was terminated. The skeletal changes in this experiment are illustrated in our photographs (Fig. 5-8) which are self-explanatory.

Discussion. It is well known that disproportionate growth of certain parts of the skeleton and localized bony overgrowths are characteristic features of clinical acromegaly. Similar changes have also been produced in rats by partially purified STH preparations (4). It had been demonstrated furthermore, that STH sensitizes the rat for the development of "topical irritation arthritis"(5), and that arthritic changes in the knee and ankle joints can be produced in gonadectomized-adrenalectomized rats, by prolonged treatment with such hypophyseal extracts(6). The observations described in this paper

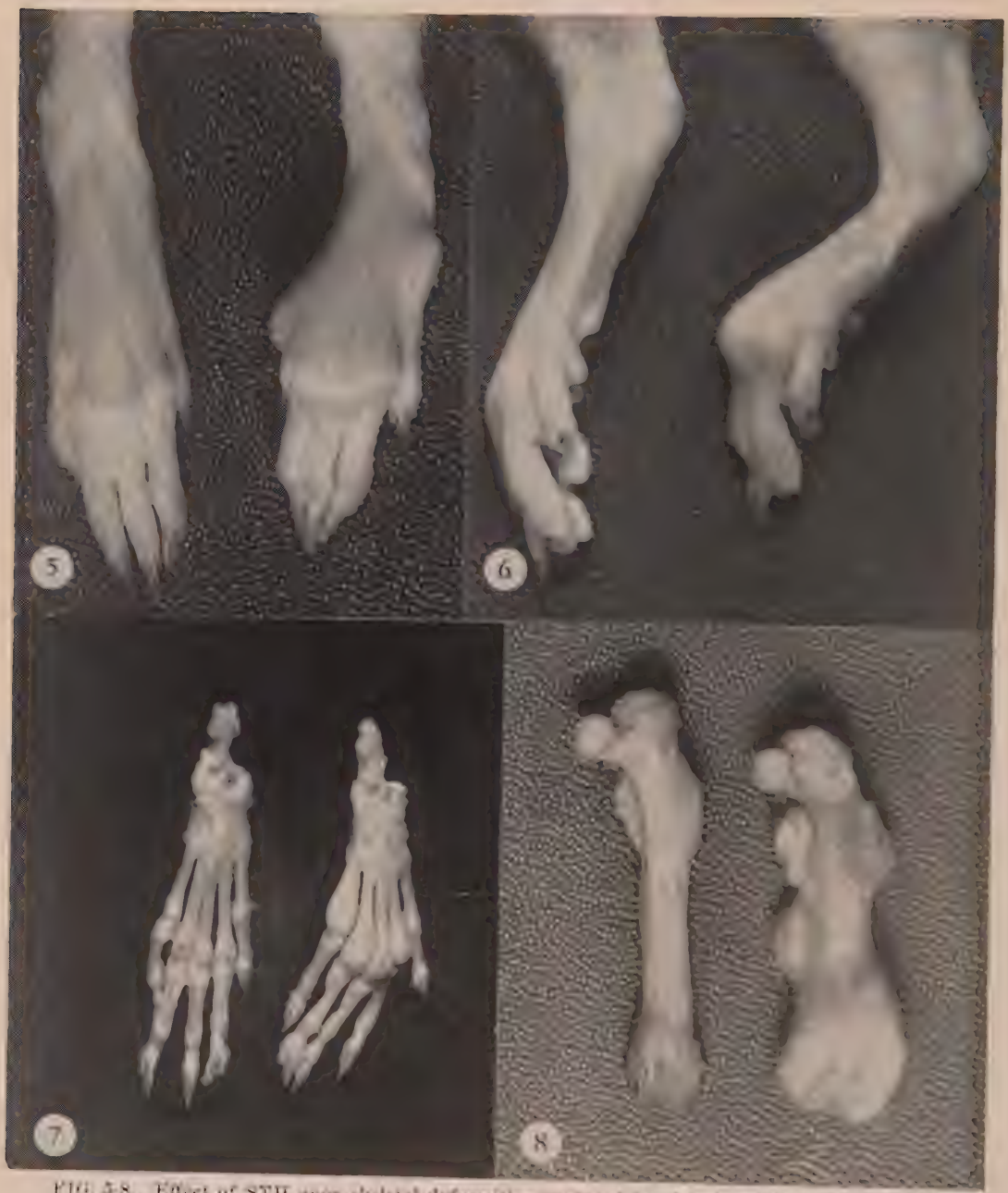

FIG. 5-8. Effect of STH upon skeletal deformities produced by chronic mild overdosage with AAN (2nd exp.).

FIG. 5. Hind paw of rat treated with AAN alone exhibits virtually normal appearance (left) while that of animal treated simultaneously with STH and AAN is greatly deformed, especially in distal parts of tarsal bones.

FIG. 6. Lateral view of paws shown in Fig. 5.

FIG. 7. Skeletal parts of paws shown in Fig. 5. Here the virtual integrity of bone of rat treated with AAN alone, and characteristic clubbing of distal ends of metatarsal bones is even more evident. Metatarsophalangeal joints are deformed and digits deviated towards the outside, as result of this abnormal growth.

FIG. 8. Anterior view of left femur of rat treated with AAN alone (left) showing essentially normal bone structure, compared with corresponding femur of animal that received AAN + STH and developed marked bone deformities. Here, growth in length is actually stunted by growth hormone.

showed that the production of skeletal lathyrism by AAN can be greatly augmented by simultaneous treatment with STH. Since neither the cartilaginous nor the bony proliferations induced by AAN are inflammatory in the usual sense of the word, this observation confirms our view that *corticoids can influence tissue reactivity to various agents, and not only to those which elicit typical inflammatory responses.* It is especially noteworthy that in the first experiment in which AAN intoxication was particularly acute and severe, concurrent treatment with STH not only failed to stimulate growth but actually inhibited it; despite this the hormone was most effective in augmenting the abnormal skeletal proliferation characteristic of lathyrism. In evaluating the possible hormonal participation in non-endocrine diseases (those not primarily due to hyper- or hypo-function of an endocrine gland) we must keep in mind that, under certain conditions, a hormone can influence a pathologic process without manifesting its classical endocrine effects.

In our opinion these observations—as well as our earlier experiments on the effect of cortisol upon AAN intoxication(1)—furnish additional support for the concept that the so-called "adaptive hormones" can participate in the development of the most diverse non-endocrine diseases. Special emphasis is laid upon the fact that this is true even of non-inflammatory tissue reactions to an exogenous irritant.

Summary. Experiments in rats indicate that somatotrophic hormone (STH) can greatly aggravate skeletal manifestations of experimental lathyrism that are produced with aminoacetonitrile (AAN). This is so even under conditions whereby STH fails to stimulate growth in length.

The authors wish to thank Dr. I. V. Ponseti of Iowa State University for the supply of Aminoacetonitrile hydrosulfate.

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Determination of L- and D-Glutamic Acids by Combined Microbiological Method and Racemization Procedure.* (22879)

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The microbiological assay of L-glutamic acid with *Streptococcus faecalis* R and of total (L- plus D-) glutamic acids with any one of several other organisms‡ has been reported previously as a provisional means of determining both L- and D-glutamic acids in protein hydrolysates(1). It has been found subsequently that utilization of D-glutamic acid by these assay organisms‡ (unpublished data)

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‡ *Leuconostoc dextranicum* (8086), *Leuconostoc mesenteroides* (8293), *Leuconostoc citrovorum* (797), and *Leuconostoc citrovorum* (7013). Numbers in parentheses refer to American Type Culture Collection catalog.

as well as by *Lactobacillus arabinosus* 17-5 (2,3) is markedly impaired in the presence of aspartic acid and that generally, therefore, the proposed method would be expected to yield erroneously low values for D-glutamic acid.§

An improved method for the determination of L-glutamic acid with *L. arabinosus* 17-5 is given in the present report. A sample treatment effecting complete racemization of glutamic acid is described as a means of estimating total glutamic acid from the L-glutamic acid content of treated material.

Methods. Assay samples, unless otherwise specified, were hydrolysed by refluxing them with approximately 10 volumes of 6 *N* hydrochloric acid for 24 hours, cooled, filtered and washed into volumetric flasks of not more than 3 times the initial hydrolysate volume, diluted with water to volume, and stored in glass stoppered bottles in a refrigerator. An aliquot of each hydrolysate to be assayed for L-glutamic acid was brought to pH 6.2 with a measured amount of standard 3 *N* sodium hydroxide solution and diluted to contain 80 to 120 γ of L-glutamic acid per ml. The diluted samples, in volumes of 0.2, 0.4, 0.6, 0.8 and 1.0 ml, were added in triplicate to 13 x 100 mm soft glass test tubes together with sufficient "blank" solution to make 1.0 ml per tube. Standard solutions of L-glutamic acid were similarly distributed to yield a 15 level standard curve ranging up to 120 γ per tube in steps of 8 γ. Standard, sample and "blank" solutions were all prepared to contain uniform concentrations of brom thymol blue (8 γ/ml) and of sodium chloride (3%, including that formed upon neutralization as estimated from the amount of sodium hydroxide employed) since this procedure eliminates possible deviations in results due to widely varying salt concentrations in the neutralized hydrolysates after appropriate dilution. The assay medium as prepared for use contained the following constituents in distilled water: vitamin-mineral mixture(4) 31 mg %, amino acid mixture‖ 1.05%, glucose 3%, buffer solution(4) 7.5 ml %, and L-glutamic acid 0.60 mg %.¶ The mixture was filtered (to remove the small amount of undissolved solids which remained after heating it briefly) and dispensed, 2 ml tube, into the sample, standard and "blank" solutions, bringing the final volume/tube to 3 ml. The pH of the medium, considerably acid owing to its relatively high aspartic acid content, was left unadjusted. The charged tubes, supported in appropriate racks and covered with aluminum foil, were autoclaved 5 minutes at 122°, cooled, inoculated with *L. arabinosus* 17-5, incubated 64 to 68 hours at 35° in a water thermostat and steamed 20 minutes in an autoclave at 100° to terminate growth. Maintenance of the stock culture(5), preparation and use of the inoculum suspension(6), measurement of growth response(4), and calculation of assay results(4) were as described in the cited references. An aliquot of each hydrolysate to be assayed for total glutamic acid was neutralized and diluted to contain approximately 2 mg of glutamic acid per ml after adding a sufficient excess of base to make the final solution 2.5 *N* with respect to sodium hydroxide. Ten-ml aliquots of the resulting solutions were heated over night (16-18 hours) at 160° in 12 ml stainless steel bombs in which the residual air had been replaced with nitrogen. The treated solutions were

§ Satisfactory recoveries of both L- and D-glutamic acids from a known mixture of pure amino acids were reported(1), but the experimental mixture contained L-asparagine in place of L-aspartic acid, and it has subsequently been found (unpublished data) that L-asparagine has relatively little effect on D-glutamic acid utilization by the test organisms.

‖ A dry mixture containing the following percentages of amino acids was homogenized in a mortar and stored in a tightly capped bottle: DL-alanine 7.5, L-arginine monohydrochloride 7.5, L-asparagine monohydrate 0.75, L-aspartic acid 49.3, L-cysteine hydrochloride 1.5, glycine 0.75, L-histidine monohydrochloride monohydrate 0.75, DL-isoleucine 7.5, L-leucine 3.77, DL-lysine monohydrochloride 1.5, DL-methionine 0.75, DL-norleucine 1.5, DL-norvaline 1.5, DL-phenylalanine 3.77, L-proline 0.75, DL-serine 1.5, DL-threonine 1.5, DL-tryptophan 0.75, L-tyrosine 1.5, and DL-valine 5.66.

¶ Insufficient to promote appreciable growth in the "blank" tubes, but sufficient to eliminate the lag which otherwise appears in the low region of the standard curve.

TABLE I. L-Glutamic Acid and Total Glutamic Acids in Casein and Various Microorganisms.

	N.	L-glutamic acid				Total glutamic acid			
		Mean	Range	M.D.M.*	No. of determinations	Mean	Range	M.D.M.*	No. of determinations
	%	%				%			
Casein	15.60†	21.3	21.0-21.9	0.9	11	21.4	21.0-21.9	1.4	6
Lactobacillus arabinosus 17-5	10.51	9.3	9.0- 9.5	1.7	7	14.2	13.8-14.5	1.2	5
Streptococcus faecalis R	10.90	11.0	10.2-11.4	3.1	7	13.7	12.7-14.1	2.6	5
Leuconostoc mesenteroides P-60	10.89	8.1	7.9- 8.3	1.3	7	14.4	14.3-14.6	.6	5
Mycobacterium phlei 10142	8.54	10.9	10.8-11.0	1.1	2	12.7	12.4-13.0	2.4	2
Mycobacterium phlei 336/289B	9.53	19.7	19.6-19.8	0.5	2	22.0	21.8-22.3	1.1	2
Mycobacterium ranae	10.94	9.8	9.7- 9.9	1.0	2	11.2	10.9-11.4	2.2	2
Mycobacterium smegmatis O. V. 66831	10.71	9.6	9.6- 9.6	0.0	2	11.3	11.1-11.5	1.8	2

Casein sample was the same as that described previously(7). Mycobacterial preparations were duplicates of those described by Ginsburg, *et al.*(13). Lactic acid bacteria were produced in 18 liter cultures (incubated 48 hr at 35°) in inoculum medium described previously(6), harvested by centrifugation, washed with 0.85% saline solution, extracted successively with boiling water, hot acetone and cold ether, and air dried. The casein was hydrolysed as described in the text, and bacterial preparations hydrolysed as described by Ginsburg, *et al.*(13). The authors are indebted to Mr. Ginsburg for preparing the mycobacteria and hydrolysing all bacterial preparations. Glutamic acid values for casein are calculated as % of moisture- and ash-free material; all others are calculated as % of protein (N × 6.25).

* Mean percentage deviation of individual values from their mean.

† Corrected for moisture and ash; all other nitrogen values are uncorrected.

cooled, transferred to separate 100 ml volumetric flasks together with sufficient brom thymol blue and sodium chloride to yield final concentrations of 8 γ per ml and 3.0%, respectively, adjusted to pH 6.2 with hydrochloric acid, diluted with water to volume, and assayed for L-glutamic acid as described in the preceding paragraphs. Total glutamic acid was represented by twice the resulting L-glutamic acid values since the described heating with alkali was sufficient to convert either L- or D-glutamic acid to an equal mixture of the two forms. D-glutamic acid was represented by the difference between the L-glutamic acid found in the untreated hydrolysates and the total glutamic acid found in the corresponding samples after heating with alkali.

Results. Previously described methods for the determination of glutamic acid with *L. arabinosus* 17-5(7-11) have not been useful with samples containing appreciable levels of D-glutamic acid because this substance under the usual assay conditions promotes a response of the test organism quite different from that effected by the L-isomer. This problem has been eliminated in the present method, however, by enriching the assay medium with aspartic acid, thereby totally inhibiting response of *L. arabinosus* 17-5 to D-glutamic acid(2,3). Earlier methods specific for the L- form of glutamic acid have been based on the use of *Steptococcus faecalis* R, which cannot utilize D-glutamic acid under any conditions thus far tested(1,8), and additional methods could presumably be based on the use of *Leuconostoc citrovorum* 8082 (1), but in the authors' experience these bacteria, neither of which either grows as profusely or produces acid as abundantly as does *L. arabinosus* 17-5, yield less readily reproducible results than the latter organism.

Reproducibility and accuracy of the present L-glutamic acid assay method are apparent from the data given in Tables I and II in

TABLE II. Recovery of L- and D-Glutamic Acids Added to Hydrolysates of Casein and *Leuconostoc mesenteroides* P-60 Cells.

Sample	Trial	% glutamic acid‖ Added	Found	Recovered¶
Casein*	1	21.45 (L)‡	42.5 (L)	98.8
	2	21.45 " ‡	42.5 "	98.8
	3	21.29 (D)	41.0 (T)	92.1
	4	21.29 "	40.1 "	87.8
L. mesenteroides P-60†	1	7.929(L)§	16.22(L)	102.2
	2	7.929 " §	15.96 "	98.9
	3	14.31 (D)	27.49(T)	91.4
	4	14.31 "	27.93 "	94.5

* Values for glutamic acid added and found are calculated as g/100 g of moisture- and ash-free casein. Recoveries based on mean values, 21.3% and 21.4%, respectively, for L- and total glutamic acids in casein (Table I).

† Values for glutamic acid added and found are calculated as g/100 g of protein (N × 6.25). Recoveries based on mean values, 8.12% and 14.41% (rounded off to 8.1 and 14.4 in Table I), respectively, for L- and total glutamic acids in *L. mesenteroides* protein (Table I).

‡ When D-glutamic acid was added at this level in place of L-glutamic acid, resulting values found for L-glutamic acid, 21.4% and 21.6%, were not appreciably different from avg value, 21.3% (Table I), found in absence of added D-glutamic acid.

§ When 8.65% D-glutamic acid was added in place of L-glutamic acid, resulting values found for L-glutamic acid, 8.2% and 8.0%, were not appreciably different from avg value, 8.1% (Table I), found in absence of added D-glutamic acid.

‖ "Added" and "found" are calculated as g per 100 g of protein (either moisture- and ash-free casein or bacterial nitrogen × 6.25). Notations, L, D, and T in parentheses refer to L-, D- and total glutamic acids, respectively.

¶ Calculated as 100 times the quotient of amount recovered (amount found in recovery sample minus that found in original sample) divided by the amount added.

which it may be seen that L-glutamic acid values from repeated assays of the same samples were in close agreement (individual deviations from the mean values averaged less than 1.4% for all repeated assays) and that recoveries of L-glutamic acid added to hydrolysates were close to 100% (average deviation from 100 was 1.4% for all recovery experiments).

Total glutamic acid values were only slightly less readily reproducible (individual deviations from the mean values averaged less than 1.5% for all repeated assays) than those for L-glutamic acid, but it is apparent from the data shown in Table II that recoveries of D-glutamic acid were significantly low (average 91.4%, range 87.8-94.5). It does not seem likely that these low recoveries were due to incomplete racemization of glutamic acid in the samples prepared for total glutamic acid determinations since virtually complete racemization of pure glutamic acid is effected within 16 to 18 hours even at 145° (Fig. 1) and the test samples were heated at 160° for this period. Moreover, if racemization of glutamic acid in the casein sample had been incomplete, erroneously high values for total glutamic acid would have resulted, and such values would necessarily have been considerably higher than the corresponding values for L-glutamic acid. Actually, as may be seen in Table I, the mean values for L- and total glutamic acids in casein did not differ by as much as 0.5%. It seems likely, therefore, that some destruction of glutamic acid in the recovery samples occurred as a consequence of the racemization procedure, even though pure glutamic acid is stable to this treatment even at 170° for prolonged periods (Fig. 1).

The value, 21.3%, for L-glutamic acid in acid hydrolysed casein (Table I) appears likely to be correct within close limits because of the apparent reliability of the L-glutamic acid assay (Tables I and II) and because of the close agreement of this value with the average value, 21.7%, found upon repeated assays of the same hydrolysate by a mano-

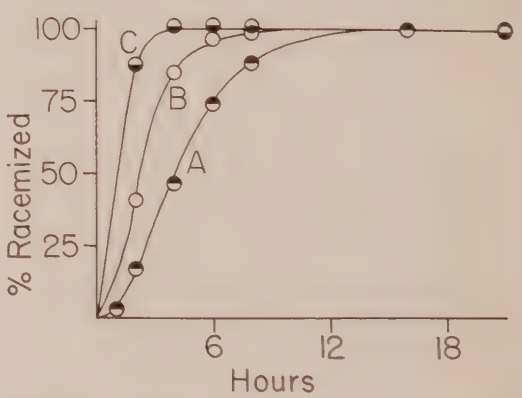

FIG. 1. Racemization of D-glutamic acid in 2.5 *N* sodium hydroxide under nitrogen at 145° (Curve A), 155° (Curve B), and 170° (Curve C). Degrees of racemization were calculated from amounts of L-glutamic acid (measured microbiologically) present in the mixtures (initially L-glutamic acid-free) after heating.

metric procedure employing a specific L-glutamic acid decarboxylase derived from *Escherichia coli*(12). The value, 20.7%, was reported earlier for L-glutamic acid in the same casein preparation(1).

It seemed likely, on the other hand, that the value, 21.4%, for total glutamic acid in the casein hydrolysate (Table I) might be somewhat low in view of the low recoveries of D-glutamic acid (Table II) and the higher values, 22.5%(7) and 22.3%(1), reported for hydrolysates of the same casein preparation analysed by procedures which would be expected to determine some or all of the D-glutamic acid in addition to the L-glutamic acid in this material. The value, 22.5%(7), might be expected to be somewhat high, however, since it was noted that *L. arabinosus* 17-5 was more sensitive in its response to racemic glutamic acid than to L-glutamic acid under the conditions employed(7), and the value, 22.3%(1), the average of values ranging from 21.3% to 23.1%, was not considered to be highly accurate. Moreover, our present value, 21.4% for total glutamic acid in casein, although 2.8% lower than that, 22.00%, reported by Chibnall and his coworkers(14), appears to be in accord with the results of these workers, quoted by Tristram(15), which indicated that only 0.7% of the total glutamic acid in acid hydrolysed casein is of the D-configuration.

The values, 9.3% and 14.2%, respectively, for L- and total glutamic acids in *L. arabinosus* 17-5 (Table I) are of the same order of magnitude as those reported earlier (6.7% and 13.1%, respectively, when recalculated as % of protein) for this species(1), although different and not strictly comparable cell preparations** were tested. The presence of a considerable amount of D-glutamic acid in cells of *L. arabinosus* 17-5 is therefore confirmed, and since the present cell preparation was subjected to extraction with boiling water, it is evident that the D-glutamic acid is tightly bound to insoluble material—probably proteins.

Significant amounts of D-glutamic acid were also found in the cells of *Leuconostoc mesenteroides* P-60 and *S. faecalis* R (Table I), an unexpected result with the latter organism, for which D-glutamic acid is neither utilizable nor capable of sparing L-glutamic acid utilization(1,8). This finding suggests that the cell wall of *S. faecalis* R is impervious to D-glutamic acid, which may nevertheless be produced intracellularly in this bacterium, probably through enzymatic racemization of the nutritionally essential L-glutamic acid.

The relative amounts of D-glutamic acid in hydrolysates of mycobacteria (Table I) are lower than in those of the lactic acid bacteria, ranging from 10% to 15% of the total glutamic acid in the former group as compared with from 20% to 44% in the latter. The values for L-glutamic acid in the mycobacteria are in close agreement (within 5%) with those reported previously for duplicate preparations(13)†† except in the case of *Mycobacterium phlei* 336/289B, for which the present value is 44% greater than that reported for the earlier preparation. The much higher percentage of L-glutamic acid in this strain than in the other strains listed in Table I is apparently correlated with its content of "cord factor"(13).

Summary. 1. An improved microbiological procedure, specific for the L- form of glutamic acid, was developed and shown to yield reproducible and accurate values for this amino acid in protein hydrolysates. 2. A sample treatment resulting in complete racemization of glutamic acid was developed to permit estimation of total (L- plus D-) glutamic acids from the L-glutamic acid content of treated materials. 3. L-Glutamic acid and total glutamic acids were determined in casein, 3 lactic acid bacteria, and 4 mycobacteria. D-Glutamic acid was negligible in casein hydrolysate but appeared to constitute 10% to 15% of the total glutamic acid in hydrolysates of mycobacteria and 20% to 44% in hydrolysates of lactic acid bacteria.

** The present preparation, but not the earlier one, was subjected to extraction with boiling water, and acetone extraction of the present material replaced the ethyl alcohol extraction which was employed in the earlier procedure.

†† Ginsburg *et al.*(13) employed the present method for the determination of L-glutamic acid, but unintentionally omitted an acknowledgement.

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Phenylketonuria VIII. Relation between Age, Serum Phenylalanine Level, and Phenylpyruvic Acid Excretion.* (22880)

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In phenylketonuria the liver enzyme system which oxidizes phenylalanine to tyrosine is defective(1); and phenylalanine accumulates in the blood(2). Phenylalanine(3) itself, and its metabolites, phenylpyruvic acid (3), phenyllactic acid(3) and phenylacetylglutamine(4) are excreted. Previous attempts to relate blood or urine levels of phenylalanine(2,5,6) or phenylpyruvic acid(6,7) to the development of the mental deficiency which accompanies the biochemical defect have not shown a significant correlation, nor have blood levels of phenylalanine appeared to correlate well with phenylpyruvic acid excretion. In the course of recent work in this laboratory it became apparent that there is a rough correlation between the level of phenylalanine in the fasting serum of patients and their excretion of phenylpyruvic acid. Also, children under the age of 3 years generally have been found to have much higher blood levels of phenylalanine than have older patients, in most of whom a rather consistent level (30 to 50 mg/100 ml) is observed.

Methods. Serum phenylalanine determinations were made on samples collected in the morning before ingestion of food; this usually has allowed a period of 15 hours for clearance of excess dietary phenylalanine from blood. Modification of the method of Kapeller-Adler (8) described previously(9) has been used for these determinations; phenylacetylglutamine and β-phenyllactic acid do not interfere. Either the method of preparation of the TCA filtrate or determination itself may have led to higher values than have been reported recently(10). Our data for normal sera are in agreement, however, with those obtained by other workers(11), and, in any event, should be internally consistent, since all determinations were carried out in the same manner. It should be noted that the method of Kapeller-Adler is inherently inaccurate when sera containing low levels of phenylalanine are assayed; at the higher levels observed with most

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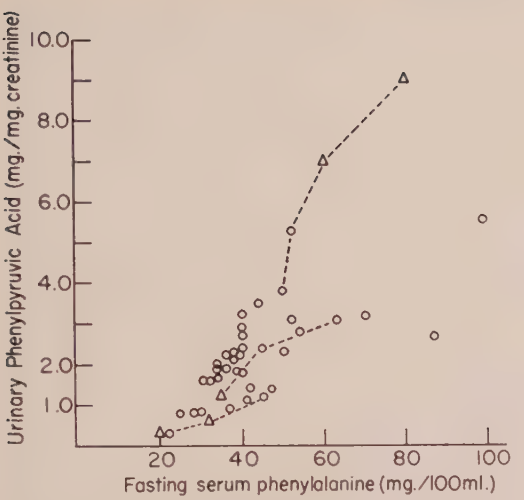

FIG. 1. Phenylpyruvic acid excretion and fasting serum phenylalanine levels of patients with phenylketonuria. △, values obtained with patients on synthetic diet deficient in or supplemented with phenylalanine. Dotted lines connect different values obtained with the same individual.

phenylketonuric patients it is sufficiently accurate to justify the conclusions made from the data.

Phenylpyruvic acid (PPA) has been determined by the following modification of the method of Penrose and Quastel(12): approximately 10 ml of urine is acidified to Congo Red by the addition of conc. HCl, stirred with 0.5 g of Lloyd's reagent, and filtered. 2 ml of filtrate (or suitable dilution) is placed in 50 ml volumetric flask, 2 ml of dinitrophenylhydrazine reagent (1.4 g dinitrophenylhydrazine in 500 ml 1 *N* HCl, warmed to dissolve, cooled, and stored in dark bottle) is added, and the mixture is allowed to stand 15 minutes at room temperature. 8 ml of 1 *N* NaOH is then added, the solution is diluted to 50 ml, filtered into a cuvette, and the optical density is measured immediately at 520 mμ. A standard curve is prepared with solutions containing from 0.01 to 0.20 mg PPA per ml; with a Coleman Junior Spectrophotometer and with cuvettes having a diameter of 22 mm these give a transmittance ranging from 88 to 11%. Determinations of PPA have been made on spot collections of urine and related to the creatinine content of the sample. The samples examined have ranged from consecutive 24 hour collections on the same patient to small samples such as those obtained from infants. The values shown in Table I represent concurrent determinations of serum phenylalanine and PPA. Values for PPA excretion obtained on different spot samples from the same patient have been quite consistent.

Results. Phenylpyruvic acid excretion. The data shown in Fig. 1 indicate a rough correlation between fasting serum phenylalanine level and amount of PPA excreted. However, a considerable variation exists in rate of PPA excretion by different individuals having approximately the same level of serum phenylalanine. Values obtained with different samples of urine from the same individual were consistent, however, and usually did not vary more than ± 0.3 mg PPA/mg creatinine. It is probable that the differences between individuals with the same serum phenylalanine level are influenced by activities of enzyme systems rather than because of differences in their intake of phenylalanine. This is indicated by data from 3 patients for whom PPA excretion was measured at widely varying phenylalanine levels; the values obtained with these patients are connected with the broken lines on Fig. 1. The younger children who might be used to confirm this by following a change in serum phenylalanine and in PPA excretion with increasing age have been maintained on a phenylalanine-restricted diet (9); it has therefore not been possible to obtain more data. The therapeutic value of phenylalanine-restricted diets for phenylketonuric infants of necessity will hamper future biochemical studies of phenylketonuria in younger children. The data shown in Fig. 1 serve to emphasize another point previously noted(9): namely, PPA is no longer detectable in phenylketonuric urine when the serum phenylalanine level falls below 15 to 20 mg %. Many samples of urine obtained from patients having serum phenylalanine levels of about 15 mg % have been examined with both the ferric chloride and dinitrophenylhydrazine tests and also chromatographically (13) for PPA with negative results. This is of significance in two regards: First, in assessing the status of patients receiving phenyl-

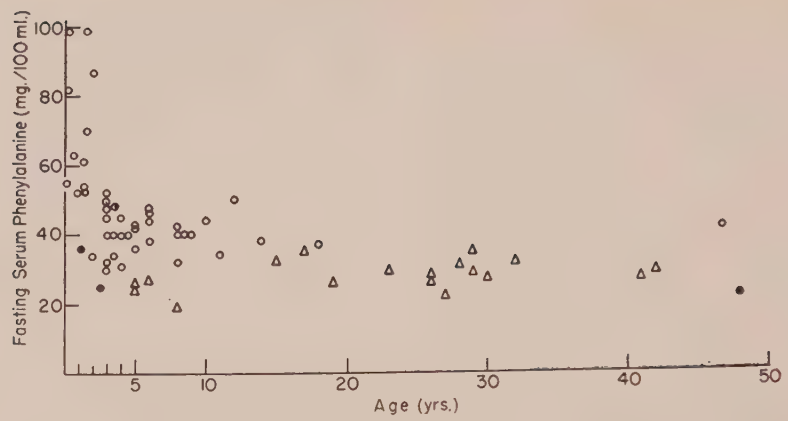

FIG. 2. Age and fasting serum phenylalanine levels of patients with phenylketonuria. ●, atypical cases commented upon in text. △, values taken from Borek, *et al.*(5).

alanine-restricted diets, a negative test for phenylpyruvic acid does not assure that an adequately low blood level of phenylalanine has been obtained, although a faint positive reaction certainly indicates 15 mg % or higher. Second, it is entirely possible that persons may exist having the trait of phenylketonuria, but with an incomplete block in the oxidation of phenylalanine so that they have higher than normal levels of phenylalanine but do not excrete phenylpyruvic acid; a case was reported recently(14) who almost belongs in this category. The only manner in which such an individual would be detected would be by a serum phenylalanine determination, or by chromatographic examination of his urine for the abnormal metabolites, *o*-hydroxyphenylacetic, *p*-hydroxyphenylacetic, and indolelactic acids.

Fasting serum phenylalanine levels. In Fig. 2 the values for serum phenylalanine in patients are plotted against the age of the patients. Very few adult phenylketonurics have been encountered in the work reported here; for values from adult patients, the data reported by Borek, *et al.*(5) have been included. They used essentially the same analytical procedure so their values should be consistent with ours. It can be seen that fairly consistent values, ranging between 20 and 50 mg% are found with patients older than 3 years. Patients younger than 3 years, however, usually show very much higher levels of phenylalanine; two children having fasting serum levels as high as 99 mg% have been observed.

Because of the considerably elevated blood phenylalanine levels, the urinary excretion of phenylpyruvic acid would be increased, as shown above. It would also follow that blood levels of phenylpyruvic acid and an increased degree of other biochemical abnormalities should occur in young children, and as a consequence, physiological and clinical symptoms should be accentuated at this age. This idea is supported by observations that electroencephalographic abnormalities are more severe in younger patients(15,16), and that abnormal liver function as manifested by the presence of abnormal β-globulins(17) or abnormal galactose tolerance curves(18) is observed more frequently in this group. In addition, observations which will be reported elsewhere have been strongly suggestive that severe damage to the nervous system probably occurs only in children under the age of 2 to 3 years.†

In regard to a possible relation between the degree of biochemical abnormalities and the severity of mental impairment in patients, it is interesting to note that the patients shown as closed circles on Fig. 2, who show lower values for serum phenylalanine than do other patients of the same age, are individuals with atypically high mental ability; two of them have been discussed elsewhere(13).

Incidence and age distribution of phenyl-

† Unpublished observations.

ketonuria in Utah. Few adult patients with phenylketonuria have been located in Utah. So far, 18 phenylketonurics born in Utah have been found; 11 of these are at present institutionalized. One is a 50-year-old man, one a 47-year-old woman, one a 20-year-old man, and the remainder are all 16 years or younger. Earlier studies in other localities have given no indication of a decrease in the incidence of phenylketonuria in older mental patients; indeed, the good physical health of most patients has been noted(19). It seems improbable that a considerable number of older patients with phenylketonuria have escaped institutionalization in Utah, hence some other reason for the increased proportion of younger patients must be sought. It seems reasonable to propose that the recent advent of sulfa drugs and antibiotics has increased the survival rate of affected children, many of whom are particularly susceptible to respiratory infections. If this is true, the older estimates of the incidence of phenylketonuria in the eastern United States of about 1 in 26,000(20) might need revision.

In Utah, there were recorded 224,576 live births from January 1, 1946 to December 31, 1955. Eleven of the patients reported in Table I were born in Utah during this 10-year period, which would give an incidence of 1 in 20,400. Since it is almost certain that all phenylketonuric children born in this period have not been detected, the present incidence of phenylketonuria in children being born in Utah must be somewhat greater than 1 in 20,000. An alternative proposal, that a different racial stock in the area might have led to this increased incidence, is controverted by the comparatively small number of older patients.

Summary. 1. The excretion of phenylpyruvic acid by patients with phenylketonuria is roughly proportional to their fasting serum phenylalanine levels. Phenylpyruvic acid is not detectable in urine of patients with serum levels below 15 mg% phenylalanine. 2. Children less than 3 years old frequently show much higher serum levels of phenylalanine than older patients. 3. The incidence of known phenylketonuria in Utah during the

TABLE I. Urinary Phenylpyruvic Acid, Fasting Serum Phenylalanine, and Age of Patients with Phenylketonuria.

Name	Age (yr)	Fasting serum phenylalanine (mg/100 ml)	Urinary phenylpyruvic acid (mg PPA per mg creatinine)
James A.†	11	34	1.9
Linda B.†	9	40	2.7
Brent C.	13 mo	54	
Robert C.	3	48	
Florence Da.†	4	45	1.2
	"	32*	.6*
	"	20*	.3*
Judy Da.†	6	46	
	8	40	2.9
Dean D.†	17		1.1
	18	37	.9
Patricia D.†	10	44	
	12	50	2.3
Allan Du.†	4	40	
	5	36	2.2
	6	38	2.3
Jill Du.†	13 mo	36	1.9
	2	34	2.0
	3	40	2.2
	5	41	1.8
Robert Du.†	7 mo	63	3.1
	8 "	17*	not det.‡
	9 "	35*	1.2*
	3	45	2.4
Mary D.	8	42	
Mary J.	10 mo	52	5.3
	10 "	10*	not det.‡
	16 "	60*	7.0*
	17 "	80*	9.1*
	3	50	3.8
John J.	4	40	2.4
David J.	3	52	3.1
Debra L.†	2½	25	.8
Anne La.†	6	44	3.5
Gary La.†	8	40	1.8
Sharon L.	3	32	1.6
Ruth L.†	47	41	1.1
Sheila M.	17 mo	54	2.8
Darryle Ma.	2 "	55	
Wauneta Ma.	4	31	1.6
Cheryl M.†	15 mo	61	
Kai N.	3	30	.8
Stephen O.	2½ mo	82	
Dennis Pr.	5	42	1.4
Norris Pr.	2	87	2.7
David R.†	13		2.5
	14	38	2.3
John S.	8	32	
Wilfred S.†	3½	34	1.7
Mary T.†	6	47	1.4
Kathy T.	18 mo	99	5.6
Clifford W.†	48	22	.3
	49		.3
Debra W.	18 mo	70	3.2
Mark Y.	4 "	99	

* Values obtained while patients were receiving synthetic diets, either deficient in or supplemented with phenylalanine. † Patients born in Utah. ‡ not det. = not detectable.

past 10 years has been 1 in 20,400 births.

We wish to acknowledge the assistance of Drs. F. A. Horner and C. W. Streamer, Dept. of Pediatrics, University of Colorado Medical Center, Denver for obtaining material from many of the patients. We are indebted to Kathryn Robinson, Patricia Wall, Eleanor Bethsold, and Lurrine Burgess for their assistance in obtaining the analytical data.

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Direct Complement Fixation by Turkey and Chicken Serum in Viral Systems.*† (22881)

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Bushnell and Hudson(1) used both complement fixation and agglutination procedures for diagnosis of *Salmonella pullorum* in chicken and turkey serum. They were able to get fixation of complement with unheated serum only. The failure to produce reactions with inactivated serum was attributed to destruction of the antibody by heat. In addition they stated that concentration of serum greater than 1/50 could not be read because of the anti-complementary quality of the serum. Rice(2) repeated this work and encountered essentially the same difficulties. She did not attribute the inability of heated serum to fix complement to destruction of antibody, but rather to the presence of inhibitory or compensatory factor in certain fowl sera. The result of this work was the development of the indirect complement fixation method(3).

In the following procedure unheated turkey and chicken sera are used in direct complement fixation. The test is qualitative in nature, designed for diagnostic work where large numbers of specimens are involved. Three viral systems have been tested, ornithosis, Newcastle disease and infectious bronchitis.

Materials and methods. The methods for direct and indirect complement fixation were essentially those of Hilleman, Haig and Helmold(4), *i.e.* all reagents were adjusted to 0.2

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TABLE I. Turkey Serum Titers in Direct and Indirect CF Psittacosis Antigen.

Flock #	Sample No.	CF	CFI	Flock #	Sample No.	CF	CFI	Flock #	Sample No.	CF	CFI
1	2	ac	ac	3	55	<1/ 8	>1/64	3	68	<1/8	0
	6	1/ 8	1/ 8		56	<1/64	1/16		69	"	0
	19	"	"		57	"	1/32		71	1/8	<1/ 8
	*	0	0		59	<1/ 8	<1/ 8		73	<1/8	>1/64
2	26	ac	ac		60	1/32	<1/32		74	"	0
	30	<1/ 8	0		61	<1/32	1/32		75	"	<1/ 8
	33	1/ 8	0		62	1/ 8	1/ 8		*	0	0
	*	0	0		64	<1/32	1/16	4	88	<1/8	<1/ 8
3	52	ac	ac		65	>1/64	>1/64		*	0	0
	53	1/32	<1/32		66	1/32	1/32	5	101-125	0	0
	54	<1/32	"		67	"	"				

Symbol < indicates reaction of 2 or 3 plus at level indicated.
* Remainder.

ml aliquots including 2% cell suspension sensitized with 2 units of hemolysin.‖ The total volume in each tube was one ml. Incubation was carried out at 37°C. The following modifications were made: (a) in place of normal saline, veronal buffered saline pH 7.4(5) containing 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$ made with double distilled water was the diluent for all reagents. (b) Both serum and antigen controls received only one-half the amount of complement used in the test and (c) 4 units of antigen were used in the direct test unless it was too anticomplementary or in case of allantoic fluid antigens when the concentrated preparation contained less than 4 units. *Antigens.* There is no fixation of complement in this system with insufficient antigen. The units are determined by titration with avian serum and strong complement explained below. Good results are obtained with a little more than 2 units of antigen, better with 4 units. For the ornithosis system,‡ crude boiled and phenolized preparations of yolk sac antigens of 6 BC strain or Jones strain ornithosis virus were used. The Newcastle antigens were Lederle's diagnostic antigen or allantoic fluid concentrated X5 in the Spinco ultracentrifuge. The infectious bronchitis antigen was also concentrated allantoic fluid which gave satisfactory results with some batches while others were unsuitable. Work is now in progress to produce a stronger and more sensitive antigen for this virus. *Sera.* Where one syringe was used for several bleedings, it was rinsed in saline. The practice of rinsing with an antiseptic caused anticomplementary effects. Serum was removed from the clot as soon as possible after arrival in the laboratory and until this was accomplished the samples were refrigerated at 4°C. In some experiments serum was replaced by citrated plasma with the same results. After samples were cleared of cells they were stored at –20°C. The time elapsing between drawing blood samples in the field and freezing seldom exceeded 24 hours. It has recently been observed that sera stored at 4°C lose their ability to fix complement. Sera for direct procedure were not heated. *Complement titrations.* The complement was pooled frozen guinea pig serum, pretested for absence of reaction with ornithosis antigen. For each new batch, the dilution to be used was established by incubating 0.1 ml (half portion) at dilutions 1/20, 1/22 and 1/24 respectively in the presence of ten unheated avian sera diluted 1/8. The hemolytic system was added after one hour and readings made 30 minutes later. The concentration of complement in 0.1 ml causing hemolysis of the indicator system was considered a half portion. Twice that amount was used for the test. Anticomplementary effects of the antigens were tested by the same method. In this laboratory screening of sera is carried out at 1/8 dilution. Un-

‖ Sharp and Dohme.

‡ We are indebted for the antigens to Dr. David B. Lackman of National Microbiological Inst., Rocky Mt. Laboratory, Hamilton, Mont. and Dr. A. L. Burroughs, Department of Veterinary Bacteriology, Texas A & M College, College Station, Texas.

TABLE II. Effect of Time on Titers of Turkey Sera in Direct and Indirect Complement Fixation.

CF			CFI		
1/25/56	6/27	7/20	1/25/56	6/27	7/20
neg.	1/ 8	<1/64	neg.	>1/64	<1/ 32
"	<1/64	"	"	"	1/ 32
"	1/16	1/32	"	"	<1/128
"	1/ 8	<1/64	"	"	"
"	<1/ 8	<1/32	"	"	>1/ 64
"	1/64	1/32	"	"	1/ 32
"	<1/64	<1/64	"	"	"

heated turkey and chicken sera were tested at this level for complement and lysins. It was found that sera frequently lysed unsensitized cells in a dilution of 1/4 and that the same or others often lysed sensitized cells in a dilution of 1/8. However, in the presence of antigen there was no evidence of zoning.

Results. The method of random sampling of turkey flocks representing approximately 10% of birds was adopted in a survey for the incidence of ornithosis in turkeys of this region. These birds were double banded and in 5 flocks showing reactors, 25 birds were later spot checked for continuing incidence. Table I shows the results for 125 samples representing 5 flocks. There is agreement between direct and indirect procedures in over 94% of the sera. Titers were higher by indirect in 2% of cases while 4% indicated higher titers or slight reactions at 1/8 dilution by direct. Although there appears to be fair correlation of titers in Table I, there is some evidence that the same type of antibody is not being measured by the 2 methods. In Table II examples of 3 sets of sera for 7 birds demonstrate the shift in titer by direct and indirect procedures as infection progresses.

Newcastle disease antisera resulted from natural infection, vaccination or both. These turkeys and chickens were experimental birds. CF and hemagglutination inhibition titers were run on the same sera. Table III shows fairly good correlation between the two tests. Citrated chicken plasma was used for titrations in Table IV. This indicates that the hemagglutinating inhibitor appears before the complement fixing antibody can be detected.

TABLE III. Turkey Serum Titers in Direct CF and Hemagglutination Inhibition NDV Antigen.

CF	HI		CF	HI
1/16	1/ 100		1/32	1/800
<1/32	1/ 200		1/16	1/400
1/32	1/ 400		"	1/100
1/16	1/ 200		"	1/200
<1/ 8	"			
1/64	1/1600	N	0	0

TABLE IV. Chicken Plasma in Direct CF and HI Tests. NDV antigen.

CF titer	HI titer	CF titer	HI titer
2/11/56		2/23/56	
<1/16	1/1600	1/ 8	1/ 400
0	1/ 100	<1/64	1/3200
0	1/ 800	>1/64	1/4000
0	1/ 200	<1/16	1/1600
0	0	<1/64	"
0	1/ 100	<1/32	"
0	1/ 200	>1/64	"
0	1/ 25	<1/32	1/4000
0	1/ 800	1/16	1/ 800
0	1/ 100	1/32	1/6400

At this time a limited number of sera have been tested in the infectious bronchitis system. It was found that the titer of the allantoic fluid must reach a level of 10^{-7} to provide 2 units of antigen. The serum was from birds vaccinated and later challenged with infectious bronchitis virus.

To compare antibody levels, the usual procedure for serum neutralization was reversed. A constant amount of virus, about 100 infectious units, was combined with 2-fold serial dilutions of serum. Table V shows the results for 5 sera.

TABLE V. Chicken Serum in Direct CF and S.N. Tests. Infectious bronchitis antigen.

	CF titer	S.N.* titer
	<1/ 64	>1/16
	<1/128	1/32
	1/ 64	1/64
	1/ 16	1/16
	1/ 32	>1/16
N	0	0

* Approximately 100 ID of virus.

Discussion. Heating certain fowl sera appears to release an inhibitory factor which alters or masks the antibody. Rice has suggested(2) the inhibitory factor may be the antibody itself which combines with antigen to form complexes with no affinity for complement. However, it has been observed that

certain turkey sera which originally reacted only by the indirect method, after several months storage, have fixed complement when inactivated. This would suggest a factor somewhat susceptible to cold storage, present in varying quantities in each serum and which in the presence of heat is capable of altering antibody. It does not appear to be free in heated serum. Where equal parts of heated immune or normal avian serum and unheated immune serum were mixed, there was neither increase nor decrease over the antibody titer of the unheated serum alone. Mixing of heated and unheated serum would also indicate that once the antibody had been altered it cannot be restored by unheated serum.

It is recognized that anticomplementary effects of serum and antigen are not additive. With fowl serum they are usually greater than the sum of the two. Proof that fixation resulted from the presence of antibody rather than the combined anticomplementary effects was tested by use of heterologous antigens of the same anticomplementary quality. Immune sera set up against heterologous antigens, within these three systems, gave negative results.

The amount of complement used in the test appears to be large for a viral system, however, the test in practice does not seem to be insensitive. The use of half portions of complement in all controls offers a margin of safety against false or nonspecific reactions.

The inability to demonstrate marked anticomplementary effects in these sera may result from viral disease as opposed to bacterial. Rice has observed(6) that prozones occur more frequently with viral antigens than with bacterial or tissue extract antigens. The level of 1/8 dilution for screening was adopted to minimize anticomplementary as well as possible lytic effects of these sera.

Although correlation of this test with other methods is not perfect, disagreement may result from measuring different types of antibody.

Summary. A procedure for direct complement fixation with unheated turkey and chicken serum or citrated plasma is outlined. Three viral systems, ornithosis, Newcastle disease and infectious bronchitis have been tested. Results are compared with other serological methods.

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Methods for Measuring Fat Absorption.*† (22882)

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A recent investigation(1) in this laboratory included a study of results from direct(2,3) and indirect methods(4,5) for measurement of rate of intestinal absorption of fat. The results from 3 indirect methods indicated a significant relationship exists between them. However, close agreement was not obtained between results of the indirect methods and a direct one, employing a modified Cori technic. Further studies have revealed that gastric retention associated with recovery of unabsorbed fat from the entire gastrointestinal tract could at times lead to erroneous conclusions, especially after relatively short absorptive periods.

Methods. Male Sprague-Dawley rats were fasted 48 hours and were administered by tube, a standard amount of fat according to their body surface. Equivalent amounts of fatty acids in the mono- or triglyceride form were fed at 2 levels of intake, equal to the fatty acids in 0.10 or 0.15 ml of olive oil. Sodium taurocholate alone and in combination with albumin were used as emulsifying agents to prevent a water-in-oil gel formation of the monoglyceride. Lipids which remained in the stomach and intestine were recovered (3) separately after a 3 hour absorptive period. This method permits calculation of the percentage of lipid absorbed on the basis of (a) the lipid which had passed through the stomach and was therefore readily available for absorption as well as on the basis of (b) the total ingested fat. Although the same amounts of fatty acid were administered in the form of 2 lipids, any retention of one lipid in the stomach would make less of it available for absorption than the other. In all cases, the lipid absorption was calculated as percentage of total fed and in mg/dm^2/hr.

Results. As can be seen in Table I, the amount of glyceride remaining in the stomach at the end of 3 hours following ingestion is greater in the case of the monooleate than the olive oil. A similar finding was obtained when intake of the 2 lipids was increased, with both lipids in an emulsified form. Hence there are marked differences in rates of absorption of the 2 lipids, whether calculated as proposed by Irwin *et al.*(2) as per cent absorbed, or by Deuel(3) in mg absorbed/dm^2/hr.

The smaller percentages of unabsorbed monoglyceride in the intestine, in contrast to the stomach, suggested that the fraction which had passed into the intestine might be as rapidly absorbed as that of the triglyceride. Hence absorption was again calculated on the basis of fat fed minus amount remaining in the stomach. Surprisingly similar values for absorption rates of both glycerides were obtained (84-89%) when only the amount actually available for absorption is considered instead of the total amount fed. Usually rates of fat absorption are considered comparable only when animals are fed similar amounts of fat, based either on amounts of fat administered or the size of the animal. However, unlike the values from the other methods at 2 levels of fat intake, similar and apparently more correct results are obtained if corrections are made for the glyceride not readily available for absorption.

The results suggest that quite misleading data may be obtained from the methods generally used for measuring relative rates of fat absorption if fat should be retained in the stomach. The retention might be the result of either some effect of the fat itself or the physical or emotional state of the experimental animal. Some correction for this unavailable fat seems necessary when comparing absorptive rates shortly after ingestion of 2 lipids which have different gastric emptying times.

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† With the technical assistance of Mrs. Joanne Blackwell.

TABLE I. Measurement of Rates of Glyceride Absorption.

No. of rats	Glyceride* and supplements	Amt fat fed, mg/dm²	% lipid recovered from		Lipid absorbed†		
			Stomach	Intestine	Abs. amt, mg/dm²/hr	% of fed	% of avail.
8	TG with bile and albumin	90.4	20	13	20.1 ± 3.4	67 ± 11	84 ± 4
8	MG emulsified with bile and albumin	103.7	44	6	16.1 ± 5.3	50 ± 11	89 ± 4
9	TG emulsified with bile and 1/5 MG	135.6	41	9	22.9 ± 7.9	51 ± 17	85 ± 6
11	MG emulsified with bile	160.3	60	6	16.2 ± 8.6	34 ± 15	85 ± 6

* MG and TG represent glyceryl monooleate and triglyceride (olive oil), respectively. The former was supplied by Distillation Products Industries.
† Including stand. dev.

These findings do not imply that both the indirect and direct methods as they are now used are not valid measurements of rate of fat absorption when gastric retention does not occur. However, the more consistent results, obtained when considering only the fat which is readily available for absorption, do suggest that a greater gastric retention in a few of a series of animals may aid in accounting for some of the variations in results. Hence separate recoveries of unabsorbed lipid from both gastric and intestinal contents should give more exact information about the absorptive process.

Summary. Gastric retention of a lipid may lead to erroneous conclusions when rates of fat absorption are measured by the generally accepted methods. Separate recoveries of the remaining lipids from the stomach and intestines after an absorptive period permits a correction for the lipid unavailable for absorption in relative rate calculations.

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Isolation of a Sulfated Mucopolysaccharide from Blood Platelets of Rats.* (22883)

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It has been observed(1) that blood platelets of rats incorporate S^{35}-labeled sulfate; and cytochemical evidence indicates that platelets contain mucopolysaccharides(2). Since it is known that sulfate becomes incorporated into mucopolysaccharides(3-5) but is not incorporated(6,7), or incorporated in only negligible amounts(8), into sulfur-containing amino acids of rats, we undertook this study to determine whether the S^{35}-sulfate in rat platelets is present in a mucopolysaccharide.

Materials and methods. Male Sprague-Dawley rats, 9-11 months old and weighing 410-538 g, were used as platelet donors. The platelets were labeled by injecting each rat intraperitoneally with 1 mc of $S^{35}O_4^{--}$ daily

* Work performed under Contract No. 7405-eng-26 for U. S. Atomic Energy Commission.

for 3 days.† Forty-eight hours after the last sulfate injection and 10 minutes after intravenous injection of 0.5 ml of a 1:10 dilution of heparin, the blood was drawn from the abdominal aorta into a siliconed syringe containing 0.5 ml of a 1% solution of Sequestrene‡ in 0.7% saline. The platelets were separated from the blood by differential centrifugation by a modification of the method of Dillard *et al.*(9). After the blood was centrifuged at 45 X *g* for 60 minutes at 5°C, the platelet-rich plasma layer was withdrawn and centrifuged at 500 X *g* for 30 minutes (5°C) to obtain the platelets. The platelets were then washed 3 times with 10-ml portions of saline and resuspended in saline to a concentration of 3.575 x 10^6 to 4.86 x 10^6 platelets/mm^3. One-ml portions of the platelet suspensions were lyophilized and stored in a refrigerator until extracted.

The sulfate-labeled platelets were extracted with 0.5 N NaOH for 7 hours at 0°C with constant stirring [modification of the method of Jorpes(10)]. The thoroughly suspended mixture was sampled for radioactivity measurement and spun at 25,000 X *g* for 15 minutes. The supernatant was sampled for radioactivity, neutralized immediately with 2 N acetic acid (dibromothymolsulfonphthalein was used as the indicator), and treated with chloroform-amyl alcohol [Sevag technic(11)] for removing protein until only a slight precipitate formed (15 to 20 times). Samples of the aqueous supernatant were taken frequently during this procedure for radioactivity measurement. The aqueous supernatant was dialyzed for 40 hours against four 1000-ml portions of distilled water, and samples were taken for radioactivity measurement. The dialyzed material was lyophilized to reduce volume and for keeping. For electrophoresis or chromatography the lyophilized material was dissolved in a small amount of 0.1 M phosphate buffer with a pH of 6.6.

Electrophoresis was carried out in a Spinco cell, Model R-Series C, Durrum type, by a method similar to that of Rienits(12). Strips of Whatman #2 filter paper 3.0 X 30.5 cm were run with 0.1 M phosphate buffer at pH 6.6 as the electrolyte for 3 hours at 10.8 V/cm (0°C). The strips were dried in an oven at 115°C, stained 30 minutes in 0.1% Alcian blue 8 GX 300§ at pH 2.5, and were washed in several changes of N/5 acetic acid and finally with water. As little as 1 μg of commercial chondroitin sulfate can be detected on Whatman #1 filter paper by this method.

Paper chromatography was carried out by a modification of the procedure of Kerby(13). Samples were applied a little at a time to sheets of Whatman #1 filter paper (15.5 by 11.25 inches), the spot being dried with a hair dryer between applications. Each spot of labeled platelet extract was measured for S^{35} activity. Each paper was then stapled together to form a cylinder and was run in an individual jar at 4°C for 32 hours, the solvent being 100 ml of a 35% solution of propanol in M/15 phosphate buffer having a pH of 6.4. The chromatograms were dried at room temperature and stained with Alcian blue.

Water lysates were prepared from platelets similarly labeled but harvested 24 instead of 48 hours after the last sulfate injection. The washed packed platelets were extracted 6 times with 1 ml of distilled water. Each mixture was spun at 1800 X *g* for 15 minutes to obtain a clear supernatant, and all supernatants were combined after the final extraction. One portion of a water lysate was treated with chloroform-amyl alcohol until only a small precipitate formed (15 times).

Samples for measurement of radioactivity were placed on flat steel planchets, air dried, and counted in an internal gas flow counter.

† $S^{35}O_4$ in weak HCl solution received from the Isotopes Division, Oak Ridge National Laboratory, was neutralized with NaOH (dibromothymolsulfonphthalein indicator).

‡ The Sequestrene (disodium ethylenediamine tetraacetic acid) was kindly supplied by the Alrose Chemical Co., Providence 1, R. I.

§ The Alcian Blue 8 GX 300 contains the same colored entity as Alcian Blue 8 GN used by Rizzoli and Gliozzi(14) and as Alcian Blue 8 GN 150, which we have also used successfully. Alcian blue 8 GX 300 and Alcian blue 8 GN 150 were kindly supplied by Arnold, Hoffman and Co., Inc., Providence 1.

TABLE I. Extraction of Radioactivity from $S^{35}O_4$-Labeled Platelets Treated with .5 N NaOH.

Extract No.	Extract mixture,* c/m/50 μl	Supernatant,† c/m/50 μl	% recovery after Extraction	Protein Pptn.	Dialysis
III	1452	1246	86	—	61
IV	2068	2096	101	100	—
V	2308	2273	98	100	45

* Comparable to whole platelets.
† After centrifuging at 25000 × *g* for 15 min.

Paper electrophoresis strips were cut into pieces 3 to 10 mm wide, placed on planchets, and counted in the same instrument. Chromatograms were counted with a large GM tube that was covered with Mylar film and gassed with Q gas.

Autoradiograms were made of some chromatograms and paper electrophoresis strips by exposing them to Kodak No-Screen X-ray safety film.

Results. From 86 to 100% of the S^{35} was extracted from the lyophilized platelets by 0.5 NaOH, and the activity remained in the aqueous fraction during the Sevag procedure for removal of protein (Table I). When the material extracted from platelets with distilled water was subjected to the Sevag technic, radioactivity was removed in proportion to removal of protein (Table II).

During dialysis of the alkali extract about 50% of the activity was lost (Table I). The S^{35}-labeled dialyzate when subjected to analysis by paper electrophoresis was found to migrate to the same place as chondroitin sulfate (CSA) and heparin or immediately behind these compounds (Fig. 1). In a mixture of CSA with the alkali extract of platelets, the labeled material and CSA migrated to the same place (CSA located by staining and the unknown by counting radioactivity).

The unknown compound was stained with Alcian blue, a dye considered to be specific for mucopolysaccharides(14).

TABLE II. Radioactivity of Supernatants during Deproteination of Distilled Water Lysates of Platelets by Chloroform-amyl Alcohol Treatment (Sevag).

No. of treatments	Radioactivity of supernatant, c/m/200 μl	% of original activity
0	3060	100
8	934	31
15	351	11

One dimensional chromatograms of the unknown compound run by a method(13) which distinguishes between CSA and heparin revealed that its mobility was similar to that of CSA but unlike that of heparin (Fig. 2).

When lyophilized platelets were refluxed with ethyl alcohol in a Soxhlet apparatus for 24 hours to remove lipids, only 5% of the radioactivity originally present in the platelets was recovered in the extract.

Discussion. It is evident that the S^{35}-labaled compound in platelets is not an alcohol-soluble lipid. In its unhydrolyzed condition, as extracted from platelets with distilled water, it appears to be associated with a protein because the radioactivity is removed in proportion to the removal of protein; however, when the platelets are extracted with alkali, an S^{35}-labeled nondialyzable compound can be separated from the protein. This suggests that the sulfur is present in a mucopolysaccharide, since alkaline extraction is a method commonly used for obtaining mucopolysaccharides from tissues(10) (*e.g.*, CSA from cartilage). In addition, after vigorous hydrolysis of platelets with HCl, radioactivity was found, upon precipitation with $BaCl_2$ (Na_2SO_4 added as carrier), to be present in $BaSO_4$ but was not present in lead sulfide when this compound was precipitated (cystine added as carrier), indicating that the activity was not present as amino acid sulfur. Furthermore, the sulfur-containing platelet material stains with a dye considered to be specific for mucopolysaccharides(14), and its migration upon paper electrophoresis is similar to that of chondroitin sulfate and heparin. Finally, when subjected to paper chromatography with a technic that separates

FIG. 1. Paper electrophoresis; line marked O indicates the origin. a, chondroitin sulfate plus platelet extract (non-dialyzable fraction); b, autoradiogram of a; c, platelet extract (non-dialyzable fraction); d, autoradiogram of c; e, chondroitin sulfate; f, heparin.

FIG. 2. Paper chromatogram; circles represent origin of the spots. a, autoradiogram of the platelet extract (non-dialyzable fraction); b, chondroitin sulfate; c, platelet extract (non-dialyzable fraction); d, heparin.

chondroitin sulfate and heparin(13), it behaves like chondroitin sulfate. However, its true identity awaits further chemical characterization.

Loss of about 50% of the radioactivity during dialysis has not been explained. The dialysis water was not analyzed by paper chromatography or by paper electrophoresis, but in one experiment it was combined, evaporated, filtered, and carrier sulfate was added. $BaSO_4$ was then precipitated by addition of $BaCl_2$. Counting revealed that 66% of the radioactivity which dialyzed out was recovered in the $BaSO_4$. This barium precipitate may be inorganic sulfate or a product of the partial breakdown of the sulfur-containing compound during alkali extraction.

Care was taken during separation of platelets from the blood to minimize contamination with white blood cells, since a mucopolysaccharide has been found in human leukocytes(15,16). In many platelet suspensions prepared as in these experiments the white blood cells have been counted and the platelet volume has been estimated to be more than 100 times that of the white cells. Hence it is not likely that the sulfate-containing material present in the platelet extracts resulted from contamination by leukocytes. Furthermore a mucopolysaccharide, removable with testicular hyaluronidase, has been demonstrated cytochemically within individual platelets separated by the technic used in this study (2).

It has been suggested that platelets may contribute to the maintenance of the interendothelial cement of the capillary walls(17), possibly by way of a platelet mucopolysaccharide. Another possibility is that the sulfonated mucopolysaccharide described here

may have either thromboplastic or antithromboplastic activity; it has been reported that hyaluronic acid has thromboplastic activity (18), whereas sulfonation of hyaluronic acid produces a strong heparin-like anticoagulant (19).

Summary. Most of the radiosulfate in S^{35}-labeled rat blood platelets was recovered in an alkali extract. The extracted material stained with Alcian blue for mucopolysaccharides and was found to behave similarly to chondroitin sulfate when subjected to paper electrophoresis and paper chromatography.

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Effect of 2-Methyl-9 Alpha Fluorohydrocortisone on Internal Distribution of Fluid and Electrolytes of Fasted Adrenalectomized Dogs.* (22884)

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It has been suggested (1-3), that animals lacking adrenal cortical hormones are unable to dilute their blood by mobilizing the water and salt in cells and tissues for transfer to the vascular system to maintain a volume of circulating fluid compatible with life. These early experiments indicated a regulatory influence of the adrenal cortex over the internal distribution of water and certain electrolytes. More recently several investigators (4-8), using DCA, cortisone and ACTH, have obtained convincing evidence for steroid induced water and electrolyte shifts between intra and extracellular compartments. The following experiments in which the potent corticoid 2-methyl-9α-fluorohydrocortisone (2-methyl FF), was employed, seem to afford unequivocal evidence that adrenalectomized dogs, exhibiting severe symptoms of insufficiency, even though deprived of food and water possess sufficient water and salt reserves in their cells and tissues to completely restore normal activity and vigor. However, they are unable to mobilize and redistribute these substances in the absence of adrenal hormones. When potent corticoids are administered i.v., water and electrolytes are withdrawn from tissues and cells in amounts adequate to (1) fully restore the failing circulation; (2) restore the normal electrolyte pattern of the blood; (3) induce renal elimination of the accumulations of K and blood urea nitrogen and (4) reduce the hemocon-

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† National Science Foundation Pre-doctoral Fellow.

TABLE I. Effect of 2-Methyl FF on Serum Electrolytes of Fasted Adrenalectomized Dogs during Recovery from Insufficiency.

Dog No.	Day bled	Dose, gamma	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	Serum electrolytes Na	Cl	K	*
									——mEq/l——			
I	1	Initial	100	21.0	13.7	36.6	5.36	86.5	138.0	111.4	4.5	N
	9	400	72	112.0	16.0	37.9	5.76	88.0	130	107.0	8.6	S
	10	1000	82	24.5	13.3	32.7	5.04	86.7				A
	11	0	90	18.0	11.4	29.8	3.10	88.0	148	117.8	3.1	R
II	1	Initial	110	20.5	12.4	32.6	4.50	80.0	144	114.0	4.6	N
	7	400	52	156.0	15.7	44.8	6.02	75.0	131	105.0	7.4	S
	8	1000	88	75.0	15.7	39.5	5.87	85.7				A
	9	0	92	30.0	13.0	39.5	5.18	86.5	144	113.0	3.8	R
III	1	Initial	112	12.0	13.47	38.0	5.34	81.5	142	121.0	4.3	N
	12	1000	62	90.4	20.44	44.2	6.46	72.5	129	103.0	7.8	S
	13	1000	86	96.4	16.23	39.7	6.13	100.0				A
	14	500	120	46.0	14.9	37.8	5.38	91.0	150	116.2	4.4	A
	15	0	120	45.5	13.61	34.3	5.85	96	147	117.4	4.4	R
IV	1	Initial	108	18.0	13.33	37.2	5.17	77.5	140	118.0	4.4	N
	10	1000	58	120.0	20.14	49.7	8.05	60.0	128	94.3	7.0	S
	11	1000	74	67.0	15.78	43.9	7.34	99.0				A
	12	500	90	39.0	14.55	39.3	5.95	102.0	141	105.0	4.2	A
	13	0	102	24.5	13.47	38.5	5.51	104.0	140	111.2	4.2	R
V	1	Initial	110	19.0	12.28	37.5	4.25	76.5	144	114	5.2	N
	8	1000	72	45.0	15.0	42.0	6.94	78.5	129	107.0	7.1	S
	9	1000	91	11.0	13.6	31.3	5.24	90.0				A
	10	0	96	10.5	11.9	30.2	4.54	80.0	136	109.0	2.4	R

* Condition of animal: N = Normal, S = Severe symptoms, A = Active and vigorous, R = Complete recovery.

centration by dilution. These changes occur within 24-60 hours regardless of additional loss of water and salt by ensuing diuresis.

Methods. The 6 dogs used had been adrenalectomized 2-5 years previously and in the interim subjected to repeated cycles of insufficiency and recovery on various types of steroid therapy. Between experiments adequate maintenance was effected by daily i.m. injections of 0.5 mg DCA in oil. The day an experiment started, DCA was withheld and the dogs sampled for study of the blood constituents. These data are included in Table I, designated as initial or control values. No further treatment was given until severe symptoms of adrenal failure became evident 7-12 days later (Table I), at which time arterial pressure and blood samples were again taken. The dogs were immediately given an i.v. injection of 2-methyl FF, in a dosage recorded in Table I, and after voiding urine, placed in metabolism cages without food or water for 48-60 hours. Injections of the steroid were repeated every 12 hours during this period, the total dose varying from 1.4 to 4 mg. The 2-methyl FF was kept as a stock solution in 100% ethanol, diluted shortly before using to 10% with warm distilled water. The total amount of fluid given as a vehicle for the compound was approximately 40 cc, which was counterbalanced by withdrawal of a similar volume of blood for sampling. Final determination of arterial pressure, serum electrolytes and other blood constituents was made at the termination of the experiment. The quantities of Na, Cl and K eliminated in the urine during the recovery period were measured and are recorded in Table II.

Results. The essential data concerning the effect of 2-methyl FF on mobilization and distribution of fluid and electrolytes are shown in Table I. The reaction of all the animals to the steroid was practically identical, the chief differences consisting in variation of the amount of water and salt excreted in the urine during revival from insufficiency. The dogs developed marked symptoms 7-12 days after discontinuing DCA (Table I), with drastic decline in arterial pressure, serum Na and Cl. Serum K rose to 7-8 mEq/l; two animals (1

TABLE II. Effect of 2-Methyl FF on Serum and Urine Electrolytes of Fasted Adrenalectomized Dogs Recovering from Insufficiency.

Dog	Serum electrolyte changes, 0-48 hr, mEq/l			Total urine electrolyte excretion, mEq/48 hr			Urine vol, 0-48 hr, cc
	Na	Cl	K	Na	Cl	K	
1	+19.0	+10.8	−5.5	14.4	33.3	46.8	835
2	+14.0	+ 8.0	−3.7	17.0	25.6	55.3	676
3	+22.0	+13.2	−3.4	5.8	16.2	54.4	534
4	+13.0	+16.9	−2.8	7.0	9.6	62.4	788
5	+ 8.0	+ 2.0	−4.6	7.5	27.5	22.5	310
6*	+ 8.0	+ 7.0	−4.0	19.0	55.0	70.0	575

+ = Increase in serum electrolyte. − = Decrease in serum electrolyte.
* Received 4 mg 2-methyl FF 48 hr.

and 3), exhibited cardiac and muscle difficulties due to hyperpotassemia. For example, Dog 1 was prostrate and unable to use his hind legs when bled. A total i.v. dose of 2-methyl FF varying between 1.4-4 mg, over 36-48 hours in 4-5 doses, restored the serum electrolytes to normal levels; in fact, the serum Na of Dogs 1 and 3 rose considerably above their initial values. Both blood urea nitrogen and serum K declined to low levels following the onset of diuresis. Hemodilution occurred as evidenced by decreases in hemoglobin, hematocrit and erythrocyte count. Blood sugar did not change significantly in Dogs 1 and 2 but did increase in those animals which received the larger doses of steroid. Coincident with restoration of normal values for the blood constituents and steep rise in arterial pressure was a striking disappearance of symptoms and resumption of normal activity. Return of vigor was obvious within a few hours after the first injection. All dogs continued to eliminate fluid and electrolytes during the fast, although the quantities of salt and water excreted showed considerable variation from animal to animal. The amount of urine voided varied from 310 to 835 cc (Table II). Potassium excretion far exceeded that of Na and Cl and the chloride exceeded sodium. Two animals (3 and 4, Table I) were fasted for 60 instead of 48 hours and received 2.5 mg of 2-methyl FF. These 2 dogs raised their arterial pressure to normal or above, whereas dogs given the lesser dosage (1 and 2) did not quite regain their initial level. Dog 6, Table II, received the largest dose of steroid or 4 mg over 48 hours. The quantity of electrolyte excreted in the urine of this fasted animal is noteworthy.

Discussion. The view(9-12), that one locus of action of adrenal cortical hormones upon Na, Cl and water metabolism is the kidney is undoubtedly correct but it is also a fact that internal transfer of these substances can readily be induced by potent steroids. The source of the fluid, Na and Cl that are mobilized is of interest. It has been suggested (5), that sodium stores ordinarily sequestered in certain tissues, *e.g.,* bone and perhaps also connective tissue(7), are made available for exchange when hormone is administered. It seems not improbable however, that all cells and tissues may relinquish a portion of their Na and water under the conditions of these experiments. A small amount of Na may be contributed by residues of unabsorbed food remaining in the alimentary tract at the time the animals were injected though most of the dogs had not ingested food for 16-24 hours before using. The quantity of Na that is redistributed may be large, for as shown in the tables, the serum Na rises to control levels despite (1) continuing loss of the cation and fluid in the urine and (2) obvious hemodilution which should lower serum Na concentration rather than increase it. The quantity of K excreted indicates that this substance was derived from cells in which it had accumulated during insufficiency and from which it was released by adrenal hormones for eventual renal excretion. The rapidity of remission of symptoms and restoration of vigor following injection of 2-methyl FF, coincident with hemodilution and reestablishment of control values for arterial pressure and serum electrolytes in the

absence of exogenous sources of fluid, Na and Cl, supports the view that one of the functions of the adrenal cortex is the regulatory control of the *internal distribution* of water and certain electrolytes between body compartments. The mechanism of action of the cortical steroids in effecting these changes remains an unsolved problem.

Summary. 2-methyl-9α-fluorohydrocortisone will revive adrenalectomized dogs from severe insufficiency and restore them to normal health within 48-60 hours even though they are deprived of food and water during recovery. The steroid fully corrects the distorted fluid and electrolyte equilibria of the blood. The lowered arterial pressure, serum Na and Cl increase to normal levels, the elevated serum K and blood urea nitrogen fall, while accompanying these changes, hemodilution occurs along with a diuresis and further loss of fluid, Na and Cl. When adrenal hormones are lacking, water and salt are not only lost in urine and other excretions but perhaps equally important, are immobilized in cells and tissues. Following administration of potent steroid ample fluid and salt are redistributed to the extracellular and intravascular compartment with complete disappearance of symptoms and return of activity and vigor.

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Adrenal Corticoid Activities of 6-Methyl-Δ^1-Hydrocortisone. (22885)

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Corticoid activity of hydrocortisone is increased by the presence of a halogen at C-9 (1,2), a double bond between C-1 and C-2 (3) and a methyl group at C-2(4,5). Potency of hydrocortisone is further augmented in some instances by the presence of two such modifications in the same molecule as in Δ^1-9-fluorohydrocortisone(6) and 2-methyl-9-fluorohydrocortisone(5). This paper reports biological activity of a new corticoid, 6-methyl-Δ^1-hydrocortisone. This steroid is about 3 times as potent as Δ^1-hydrocortisone in the glycogen deposition assay and twice as potent in the anti-inflammatory assay. It has no measurable sodium or water retention activity —on the contrary, it causes diuresis of sodium and water in the test animal.

Materials and methods. Steroids. Steroid suspensions were made by grinding the compounds with carboxymethylcellulose (CMC) vehicle(6) in a ground glass tissue homogenizer. Compounds used were: hydrocorti-

sone, 11β, 17α, 21-trihydroxy-4-pregnene-3,20-dione (F); Δ^1-hydrocortisone, 11β, 17α, 21 - trihydroxy-1, 4-pregnadiene - 3,20-dione (Δ^1-F); desoxycorticosterone acetate, 21-hydroxy-4-pregnene-3,20-dione, 21-acetate (DOCA); and 6-methyl-Δ^1-hydrocortisone, 11β, 17α, 21-trihydroxy-6-methyl-1, 4-pregnadiene-3,20-dione (6-methyl-Δ^1-F). The latter steroid was recently reported from our laboratories(7).

Animals. Rats from the Upjohn colony (Sprague-Dawley ancestry) were used in these experiments. In the glycogen deposition and sodium retention assays, male rats were adrenalectomized at a body weight of 140-160 g and fed stock laboratory diet (Purina Laboratory Chow) and 1% NaCl as a drinking fluid. In the anti-inflammatory assay, the test animals were intact female rats weighing 150-160 g.

Anti-inflammatory test. The granuloma pouch technic of Selye(8) as modified by Robert* was used to assess anti-inflammatory activity. In this assay, 25 ml of air are injected subcutaneously into rats, followed by 0.5 ml of a sterile 1% solution of croton oil in cottonseed oil. On the second day after formation of the pouch, the air is removed by vacuum; on the third day the pouch is compressed manually to prevent the formation of adhesions; on the fourth day the pouch is opened and the exudative fluid is measured in graduated cylinders. The test measures the ability of steroids to inhibit inflammatory exudation of fluid into the granuloma pouch.

Glycogen deposition test. This assay for glucocorticoid activity is a modification(6) of the method of Pabst, Sheppard and Kuizenga(9).

Sodium retention test. This test(6), based on the method of Marcus, Romanoff, and Pincus(10) measures the effect of compounds on sodium excretion in the adrenalectomized, salt- and water-loaded rat within 4 hours after administration.

Results. Fig. 1 depicts the comparative activities of 6-methyl-Δ^1-F, Δ^1-F and F in inhibiting fluid exudation into the granuloma pouch of rats following subcutaneous injection of the steroids suspended in .2 ml CMC vehicle. These data indicate the potency of 6-methyl-Δ^1-F to be about 6 times F and twice Δ^1-F by this route of administration.

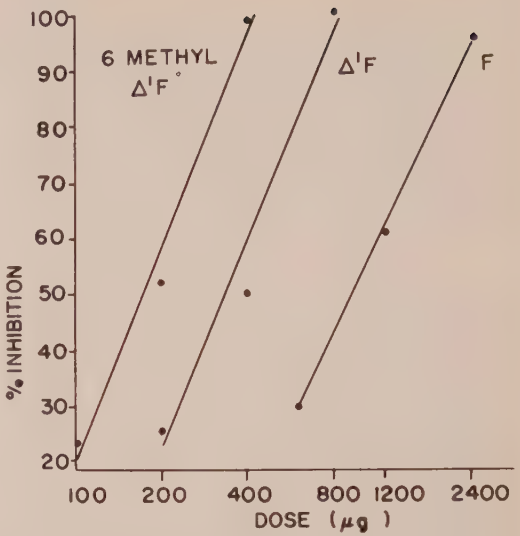

FIG. 1. Effect of F, Δ^1-F and 6-methyl-Δ^1-F in inhibiting fluid exudation in granuloma pouch following subcutaneous administration of steroids. Each point is the mean value for 6 or 7 rats.

6-Methyl-Δ^1-F and Δ^1-F were each compared with F for liver glycogen deposition activity by both subcutaneous and oral routes. 6-Methyl-Δ^1-F was found to be about 10 times as potent as F subcutaneously and 16 times F orally, and Δ^1-F to be 3 times F subcutaneously and more than 5 times F orally (Table I). 6-Methyl-Δ^1-F is therefore cal-

TABLE I. Oral and Subcutaneous Glycogen Deposition Activity of F, Δ^1-F and 6-Methyl-Δ^1-F. Potency ratios calculated according to Irwin(11).

		Liver glycogen deposition activity Potency ratio	
		Δ^1-F	6-Methyl-Δ^1-F
No. rats	Route of admin.	F	F
30	Oral		18.5
30			18.1
45		4.4	11.6
45		6.3	16.4
Mean		5.4	16.2
30	Subcutaneous	2.2	
30		3.6	
30		2.7	
30			10.6
30			8.5
Mean		2.9	9.6

* Personal communication.

TABLE II. Comparison of Effects of 6-Methyl-Δ^1-F and DOCA on Sodium and Water Excretion in Adrenalectomized Salt-Loaded Rats. Six rats were used for each determination.

Compound	Dose (μg)	Urine vol (cc)	Urine sodium (mg)
None		6.1	28.5
DOCA	10	6.9	24.9
	20	5.8	21.5
	40	4.9	16.0
6-Methyl-Δ^1-F	10	8.8	35.6
	50	10.6	35.2
	100	12.0	37.6
	200	11.2	32.2
	500	11.8	33.0
	1000	11.3	30.5

culated at about 3 times the potency of Δ^1-F by either route of administration. Both steroids show enhanced oral:parenteral activity ratios when compared with F. Table II presents data obtained from the subcutaneous injection of 6-methyl-Δ^1-F and DOCA in the sodium retention assay. 6-Methyl-Δ^1-F not only failed to induce sodium and water retention, but actually acted as a diuretic and natriuretic agent in the adrenalectomized rat.

Discussion. Chemical modifications of F frequently alter glucocorticoid, anti-inflammatory and mineralocorticoid activities in an unpredictable manner. Table III is a summary of data obtained in this laboratory from testing 6 analogues of F, each with one or two modifications present in the molecule. It will be noted, for example, that fluorine at C-9 increases sodium retention activity to a far greater degree than it does glucocorticoid potency, while in contrast, a double bond between C-1 and C-2 enhances the latter without affecting electrolyte activity. It is also of interest that whereas 2-methyl-9α-fluorohydrocortisone has only about 70% the glucocorticoid and anti-inflammatory activity of Δ^1-9α-fluorohydrocortisone, it is at least 20 times more potent in retaining sodium.

The electrolyte activity of these analogues is of special interest. Although the analogues of F with fluorine at C-9 or a methyl group at C-2 have greater anti-inflammatory potency than F or its Δ^1 analogue, their remarkable salt retaining activity limits clinical usefulness as therapeutic agents in inflammatory diseases. 6-Methyl-Δ^1-F, on the other hand, has increased glucocorticoid effectiveness as compared with F or Δ^1-F without exhibiting salt retention activity. In fact, the diuresis of sodium and water in rats treated with this steroid deserves comment. Animals receiving 100 μg doses of 6-methyl-Δ^1-F doubled the excretion of urine and increased urinary sodium loss by 30% as compared to untreated rats. Since F fails to cause sodium retention in our test animals contrary to its effect in man, it can be presumed that this assay may exaggerate the salt losing activity of 6-methyl-Δ^1-F. The natriuretic activity of this new analogue of F will be the object of further investigation.

Summary. Comparisons are presented on relative potencies of Δ^1-F and a new analogue, 6-methyl-Δ^1-F, on 3 different assays for corticoid activity: anti-inflammatory (granuloma pouch technic), liver glycogen deposition, and sodium retention. 6-Methyl-Δ^1-F is 2 times as potent as Δ^1-F on the anti-inflammatory test and about 3 times Δ^1-F on

TABLE III. Comparative Corticoid Activities of Analogues of Hydrocortisone.

Steroids	Glycogen deposition (× hydrocortisone)	Anti-inflammatory* (× hydrocortisone)	Sodium retention (× DOCA)
Hydrocortisone	1	1	Very slight activity†
Δ^1-Hydrocortisone	3†	3	*Idem*
6-Methyl-Δ^1-hydrocortisone	10	6	Na and H_2O diuresis
9α-Fluorohydrocortisone acetate	13†	7‡	5†
Δ^1-9α-Fluorohydrocortisone acetate	49†	14‡	5†
2-Methyl-hydrocortisone acetate	10§	5§	3§
2-Methyl-9α-fluorohydrocortisone acetate	38§	9§	90§‖

* Cotton wad technic used except for assay of Δ^1-hydrocortisone and 6-methyl-Δ^1-hydrocortisone. † Stafford *et al.*(6). ‡ Dulin(12). § Byrnes *et al.*(5). ‖ More recent testing in this lab indicates somewhat greater potency.

the glycogen deposition assay. 6-Methyl-Δ^1-F, like Δ^1-F, does not induce sodium and water retention on the assay for electrolyte activity, but in contrast to Δ^1-F, this steroid acts as a diuretic and natriuretic agent.

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Reaction of Splenic Tissue in Culture to *Listeria monocytogenes*. (22886)

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(Introduced by C. K. Whitehair)
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A study of *Listeria monocytogenes* in tissue culture was conducted because of the peculiar behavior and wide host susceptibility in experimental animals(1,2) as well as in natural hosts(3-8). Listeric infections in rabbits cause a monocytosis with a generalized infection while ruminants usually manifest a severe encephalitis. Abortions due to *L. monocytogenes* have been reported in cattle, sheep, goats, swine and rabbits(9,7). In recent years several reports of abortion, and early deaths in infants due to *L. monocytogenes* have come from both sectors of Germany(10-12). The disease has been called *granulomatosis infantiseptica* and is characterized by a granulomatous reaction associated with capillary endothelium(10,13). Gray *et al.*(14) reported that maceration of tissues followed by refrigeration increased the probability of recovery of the organism from infected tissues; other workers have reported similar results. Because of this strange behavior inoculated tissue cultures were studied for this same phenomenon.

Materials and methods. The culture of *L. monocytogenes* used was isolated from the brain of a calf with listeric encephalitis. Previously, Gray *et al.*(16) reported that the concentration of bacteria obtained from a suspension made to match the 0.5 tube of a MacFarland nephelometer was adequate to produce symptoms and lesions typical of *L. monocytogenes* infection in rabbits. This concentration of bacteria did not suppress growth of tissue in culture. The bacterial suspension was prepared by washing the 24-hour growth off tryptose agar (Difco) slant with sterile saline. This suspension was diluted with saline until the density matched the 0.5 tube of a MacFarland nephelometer when read in a Cenco Sheard Sanford photelometer. An inoculum of 0.01 ml of this bacterial suspension was added to the tissue cultures from tip of 27 gauge needle. The double coverslip method of Maximow(15) was used throughout. Explants of adult rab-

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FIG. 1. Inoculated culture after 24 hr incubation. Note beginning of proliferation of vascular endothelium.

bit spleen were placed in a plasma clot and the nutrient medium consisting of 60% solution 199, 20% embryo extract and 20% rabbit serum was added. The cultures were incubated as a lying drop at 37°C. All cultures were examined daily for cell migration and outgrowth. Uninoculated controls were compared with cultures which had been inoculated with the bacterial suspension. For histopathological studies, inoculated and control cultures were removed daily and fixed in Bouin's fluid. Serial sections were made of the entire explant and outgrowth, stained with hematoxylin-eosin, and Weigert-Van Gieson for connective tissue determination. To test the viability of *L. monocytogenes* in tissue culture, the inoculated explants were removed from culture slides, placed in tryptose broth and incubated at 37°C. To study the effect of refrigeration on exposed explants, some tissue cultures were held at 4.0°C while still in Maximow slides according to the following schedule. After 4 days at 37°C, 6 tissue cultures were removed from incubator each day for 4 consecutive days and placed in refrigerator (4.0°C) for periods of 7, 16, 23, and 31 days. Then each group of 6 cultures was unsealed, placed in tryptose broth and incubated at 37°C for at least 2 weeks.

Results. Living cultures. During the first 24 to 40 hours following incubation, polymorphonuclear leucocytes migrated outward from the explant, gradually became inactive and degenerated. After 2 days large, ameboid, phagocytic cells were seen to move slowly in a random manner. Bacteria were distributed diffusely throughout the cultures.

Fixed cultures. Explants which were removed daily for 6 consecutive days and sectioned serially revealed definite pathological changes. The reaction began by what appeared to be a proliferation of the vascular sinus endothelium. As early as 24 hours, two layers of endothelial cells were seen circumscribing a small vessel. Radiating from this blood vessel were single rows of cells infiltrating the surrounding tissue (Fig. 1). The nuclei of these cells were vesicular, elongated

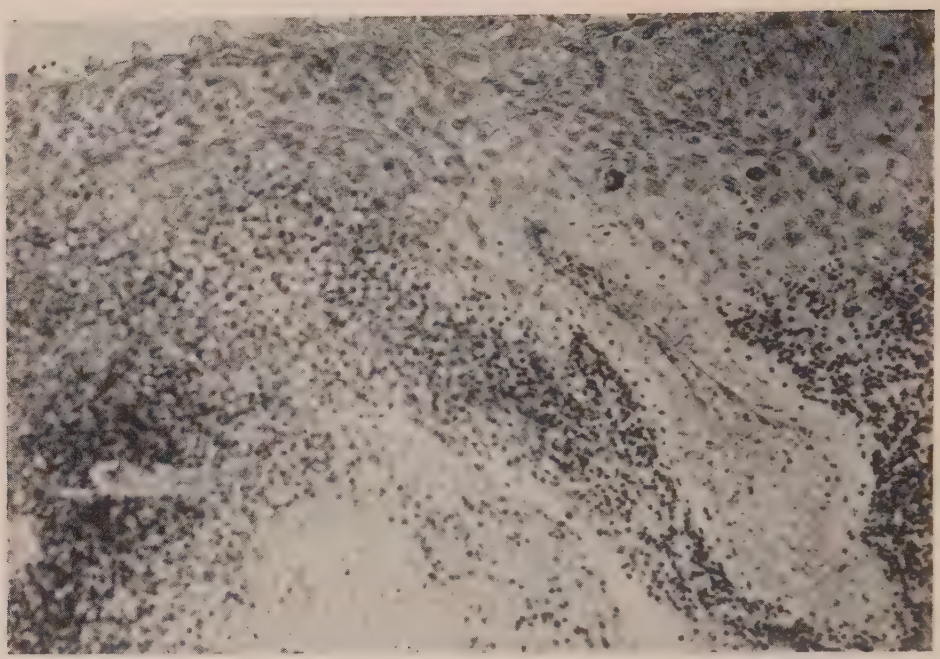

FIG. 2. Inoculated culture after 6 days incubation. Note formation of granuloma as a result of cellular proliferation.

and pleomorphic. As a result of the continued proliferation of the endothelium, the cells appeared as swirls around the vessels until the involved area was of considerable size at the end of the 6-day period and took on the characteristics of a granuloma (Fig. 2). The epithelioid cells which made up the granuloma, or cell mass, always circumscribed a vascular sinus. Sections stained with Weigert-Van Gieson connective tissue stain revealed elastic fibers around the lumen of the vessels in the center of the proliferated cells. Exceptionally large, round macrophages were seen at the margin of this cell mass after at least 4 days. They appeared to be identical with the cells of the granuloma, but were more spherical and usually contained a yellow pigment. Attempts to associate the presence of the bacterium with this pigment failed.

Uninoculated control cultures did not exhibit any proliferative cellular reaction. There was some necrosis at the center of the explant due to the inability of the nutrients to penetrate the explant (Fig. 3).

Refrigeration. When a 24-hour explant was unsealed, and placed in tryptose broth, *L. monocytogenes* grew luxuriously after 18 hours incubation. However, when exposed explants were incubated 48 hours or longer and then cultured there was no evidence of bacterial growth even after 1 week incubation at 37°C. Nevertheless, if these exposed explants were placed in the refrigerator for periods of 7, 16, 23, and 31 days, *L. monocytogenes* was recovered from broth cultures after 48 hours of incubation at 37°C.

Discussion. Histopathological study revealed a reaction definitely associated with the vascular endothelium. It is questionable that these cells are true monocytes, but they have characteristics which are associated with mononuclear phagocytes. Also, it should be noted that *L. monocytogenes* causes a very severe monocytosis in rabbits. It has been established that the vascular sinus endothelium has been considered a part of the reticulo-endothelial system. However, considerable controversy remains regarding the origin of the mononuclear phagocyte. Forbus (17) stated that the mononuclear wandering cells are derived either from primitive mesenchymal cells or from vascular sinus endothelium, with the latter being the theory most widely accepted. It is of interest that the

FIG. 3. Control culture after 4 days incubation. No proliferative cellular reaction.

granuloma formation is similar to that reported by German workers(4,10,13) describing the pathology associated with *granulomatosis infantiseptica*. It is their belief that the granuloma is derived from vascular sinus endothelium.

It is possible only to speculate on the phenomenon of increasing recovery of *L. monocytogenes* following a period of refrigeration. Perhaps some inhibitory substance acts as a bacteriostatic agent at 37°C, but when refrigerated, no longer retains this bacteriostatic property. Another possibility is that some biological system may become inactivated at 4°C allowing the bacteria to grow on artificial media.

Summary. Serial sections of infected tissue cultures revealed a granuloma due to the proliferation of the vascular sinus endothelium. This reaction was similar to that reported by German authors in describing lesions of *granulomatosis infantiseptica,* a disease caused by *L. monocytogenes.* Infected tissue cultures incubated 48 hours or longer required refrigeration for at least 7 days in order to demonstrate the presence of *L. monocytogenes.*

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Mitotic Activity of Mast Cells. (22887)

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Michels(1) in his review of mast cells comments "practically without exception investigators who have encountered mitotic dividing mast cells have stated that instances of them are extremely rare." This opinion has apparently not changed in recent years judging by comments made by Asboe-Hansen(2) at Josiah Macy, Jr. Foundation Conference on connective tissues. On the other hand mast cells are often seen in what appear to be isogenous groups as shown here in Fig. 7 and it has been assumed(3) that mitosis or amitosis has occurred. Direct evidence has been lacking, however. That cell division has been observed rarely in mast cells does not necessarily mean that it does not occur even with considerable frequency. Observations may have been made at the wrong time or under unfavorable conditions or the division figures overlooked. As usually stained with toluidine blue, nuclear material is either obscured by heavy granulation or is relatively colorless if the stain has been acidified. This latter procedure facilitates the identification of mast cells but greatly reduces the chances of seeing mitotic figures.

In the present study, a considerable number of mitotic figures were observed in mast cells of the rat following injection of the histamine liberating compound 48/80.

Materials and methods. A Long-Evans strain of male rats 100 days old or more was used. Animals were injected intraperitoneally with the histamine liberator, compound 48/80.* Rats in Group 1 were given 100 γ daily (concentration of 1000 γ/ml of saline) for 7 days and then were killed either on 8th day or in a few cases on 16th or 24th day. Those in Group 2 had 500 to 1000 γ daily for the same length of time and were killed on the 8th day. At time of death preparations of mesentery and subcutaneous films of connective tissue of the back were made by stretching the tissue over Bristol board rings with punched openings about 15 mm in diameter. Preparations were fixed in cold formol and absolute ethanol (10:90) and stained in 0.1% toluidine blue which in most cases was not acidified. After dehydration and clearing, the tissue was cut from the ring and mounted.

Observations. In Group 1 (with the smaller dosage) the 48/80 was sufficient to result in disappearance of the large-sized, heavily granulated mast cells usually present in the mesentery. There remained, however, a considerable although variable number of medium-sized cells having diameters of 14 to 24 μ with fewer granules. Among these cells mitotic figures could be readily observed (Fig. 2 to 5).

It is difficult to give an accurate quantitative count of the mast cells in division because the mitotic figures are relatively widely and unevenly spaced and are easily overlooked. In one preparation of mesentery, however, covering approximately 12 square mm, 6 mitotic figures were seen among a total of 364 mast cells. In another preparation 2 mitoses were seen in one high power field. In other preparations dividing cells were more widely spaced but could be found with careful searching.

* The compound 48/80 was kindly supplied by Dr. E. J. deBeer of Wellcome Research Laboratories.

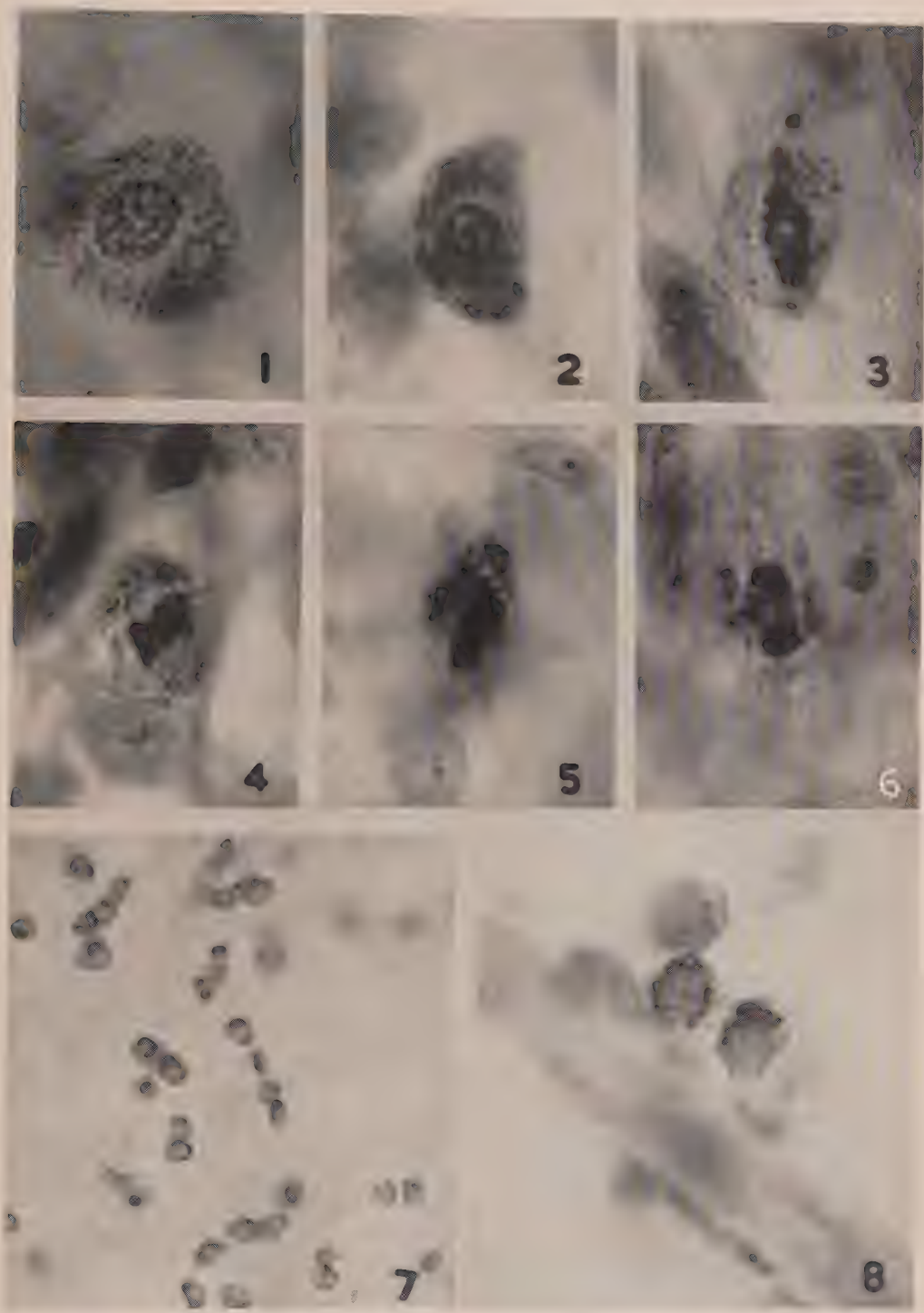

FIG. 1. Mast cell in prophase in subcutaneous connective tissue. ×1400.
FIG. 2-5. Mitotic figures in mast cells of mesentery. ×1400.
FIG. 6. Mast cell in telophase in subcutaneous connective tissue. ×1400.
FIG. 7. Isogenous groups of mast cells in subcutaneous connective tissue. ×380.
FIG. 8. Mast cells along capillary of omentum; rounded ones above vessel, elongated one below vessel. ×1400.

In animals receiving heavier doses of 48/80 no mast cells remained in the mesentery presumably because of their disintegration. In subcutaneous connective tissue, however, they were affected only moderately and while the larger 20 to 30 μ, heavily granulated cells were no longer present as such, there were considerable numbers of smaller ones 12 to 15 μ in diameter. In one preparation a total of 8 mitoses was seen (Fig. 1 and 6). In some regions many isogenous groups of 2, 3 or more cells occur (Fig. 7). These cells, having an average diameter of 10 μ, are believed to have divided recently and have not yet had time to increase in size. Some mitoses were also found among these cells but the nuclear material was stained so poorly as a rule that mitotic figures could not often be identified with certainty.

In animals that were killed 9 days after the smaller doses of 48/80, the mast cells of the mesentery had again become heavily granulated and by the 17th day most had reached a diameter ranging from 16 to 20 μ.

Discussion. In previous investigations(4, 5) the conclusion was reached that cells produce a mitotic inhibiting substance which when diminished sufficiently allows mitosis to occur. The diminution may occur after strong secretory activity as well as after death of some of the cells in the area. In the case of mast cells the 48/80 causes a diminution of the product of mast cells, histamine, from the vicinity of the cells(6). Fawcett(3) has shown that a moderate amount of 48/80 results in a release of granules followed by rapid cellular recovery. The released granules, which may include the mitosis inhibiting substance as well as histamine, are rapidly removed from the region of recovering cells by phagocytic activity of macrophages(3) or fibroblasts(7) or both, and consequently conditions are then realized which will activate cell division.

Mast cells may be affected to varying degrees by various stimulating substances such as 48/80 so that there may be a range from mild response to complete disintegration of mature cells in a region such as the mesentery. With mild stimuli the cells are believed to pass through a secretory phase without division. With a stronger stimulus as given in the present experiments the cells are more nearly exhausted and cell division occurs. With still stronger doses disintegration of the cells occurs and replacement then may follow by differentiation from stem cells.

The development of mast cells from stem cells is comparable to heteroplastic hemopoiesis. In the lymphatic milk spots of omentum or in their vicinity, there are many small mast cells only 5 to 8 μ in diameter (Fig. 8) having many of the characteristics of medium-sized lymphocytes. Also along the small blood vessels are irregularly shaped, small mast cells such as Riley(8) described. These cells, which are much smaller than fibroblasts of the vicinity, are believed to have differentiated from mesenchymal cells. Their number appears to have increased after 48/80 but this is difficult to prove because they vary so much in frequency in control animals. They do not release their granules even with the largest doses of 48/80.

Fawcett(3) in describing pairs of mast cells after moderate doses of 48/80 considers that they have appeared probably due to cell division, but he does not offer an opinion or evidence to support the statements by others that division occurs either by mitosis or amitosis. Except for the occurrence of what appear to be isogenous groups there is little or no evidence that amitosis of mast cells occurs *in vivo*.

The percentage of mast cells undergoing division at any one time probably varies greatly just as it does in other types of cells, and the number depends on mitotic stimulating factors. In the one instance in which a quantitative count was attempted, 1.6% of the mast cells were found in division. In organs such as liver, kidney and adrenal gland, Stevens and Leblond(9) state that only 0.1% of the cells are in division at any one time. Thus on a comparative basis the occurrence of mitosis in mast cells of mature animals is no more infrequent than it is among other cells where the occurrence of mitosis is unquestioned.

Summary. 1) Mitotic activity occurs in

adult mast cells of the mesentery of rats after moderate intraperitoneal doses of the histamine liberator, compound 48/80. After larger doses, which cause disintegration of mesenteric mast cells, mitoses are found in mast cells of the subcutaneous tissue. 2) Replacement of cells after disintegration appears to be by heteroplastic differentiation of lymphocytes or undifferentiated mesenchymal cells or both.

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Co^{60} Vitamin B_{12} Binding Capacity of Normal Human Serum.* (22888)

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Recent studies(1-4) of plasma concentration and transport mechanisms of vit. B_{12} have included microbiological measurements of its serum *in vitro* binding capacity. Results have varied widely. The present investigations are concerned with estimation of Co^{60} vit. B_{12} serum binding capacity.

Methods. Sera were obtained from 40 normal persons. The Co^{60} vit. B_{12} had a specific activity of 0.786 mc/mg. Its physiological activity was proved when 3 μg given intravenously to a patient with pernicious anemia in relapse induced a moderate reticulocyte response and clinical improvement. To 1 ml of serum was added an aliquot of Co^{60} vit. B_{12} ranging from 1 to 500 μg. This was done either by adding the chosen amount of vitamin directly, or by making an initial concentrated solution in serum, then rediluting with more serum at once. These methods gave equal results. Specimens were prepared in duplicate, one to serve as a standard. Each was pipetted into a Visking bag. Samples were incubated at room temperature for 2 hours, then subjected to constant agitation and exhaustive dialysis in cold, running tap water for 48 hours. Standards were placed in 4 ml vials at once; samples were placed in similar vials at conclusion of dialysis. Each vial was filled with concentrated sulfuric acid, dissolving the bag and allowing complete mixing within the vial. Counting was done in a well-type scintillation counter. "Bound" vit. B_{12} was calculated as activity retained in the bag after dialysis. Parallel studies were made with $Co^{60}Cl_2$ and sera from 14 normal subjects. The specific activity and cobalt content of the $Co^{60}Cl_2$ solution were equal to that of the radioactive vit. B_{12}. Continuing dialysis for 72 or 96 hours did not decrease residual activity further than the 48-hour period used. Incubation of the serum-B_{12} mixture for more than 2 hours prior to dialysis did not alter amount of vit. B_{12} bound. Dialysis for 48 hours in a reservoir of normal saline, 6% dextran in normal saline, or human plasma 50x the volume of the samples and changed at 24 hours gave the same results as exhaustive dialysis in tap water. Co^{60} B_{12} in saline dialyzed for 48 hours in tap water showed residual activity within the Visking bag of less than 2% at any concentra-

*Co^{60} Vit. B_{12} was obtained from Merck & Co., Rahway, N. J.

TABLE I. Co^{60} Vitamin B_{12} Binding Capacity of Normal Human Serum.

mμg $Co^{60}B_{12}$ added per ml serum	No. of sera tested	% $Co^{60}B_{12}$ bound ± 1 S.D.	mμg $Co^{60}B_{12}$ bound	Serum protein, g %	mμg $Co^{60}B_{12}$ bound per g serum protein
1	23	88.5 ± 13.1	0.89	6.9	12.8
2.5	23	67.4 ± 6.3	1.69	6.9	24.4
5	23	42.9 ± 3.9	2.15	6.9	31.1
10	10	34.0 ± 5.2	3.40	6.7	50
25	10	24.3 ± 4.8	6.08	6.7	101
50	10	22.7 ± 2.4	11.35	6.7	168
100	7	26.3 ± 3.2	26.3	7.5	352
250	7	26.3 ± 4.8	66.6	7.5	878
500	7	26.0 ± 2.5	130	7.5	1743

TABLE II. $Co^{60}Cl_2$ Binding Capacity of Normal Human Serum.

Amt $Co^{60}Cl_2$* added per ml serum	No. of sera tested	% $Co^{60}Cl_2$ bound	Amt $Co^{60}Cl_2$ bound
1	6	84.2	0.84
2.5	6	88.0	2.2
5	6	85.8	4.3
10	2	89.0	8.9
25	2	90.0	22.5
50	2	85.5	42.7
100	5	76.0	76

* Contains Co in equal amounts to corresponding aliquots of $Co^{60}B_{12}$.

tion. When the bag was opened and washed following dialysis of the B_{12}-serum, activity was found on the casing ranging from 8% of the total residual activity at the lowest concentration of vitamin to 3% at the highest. No correction was made for this activity.

Results. Table I shows that as the vit. B_{12} aliquot was increased, the fraction bound fell to a plateau while the absolute amount rose. The highest amount bound was 130 μg/ml serum. The standard deviation at each level showed a narrow range of individual binding capacities. Calculated binding capacity per gram of serum protein paralleled that of whole serum.

Analogous studies on serum binding of $Co^{60}Cl_2$ are shown in Table II. A high percentage is bound at all concentrations. These data are shown graphically in Fig. 1 and 2.

Discussion. Bound vit. B_{12} is defined by microbiological assay as that portion of the vitamin which becomes available to the organism only after the B_{12}-serum is heated to 100°C for 30 minutes. Rosenthal(1), using *Lactobacillus leichmannii* for assay after dialyzing the B_{12}-serum 48 hours, showed bindings in mixtures of 2 and 6 mμg of vit. B_{12} per ml serum similar to those in the present study. However, when as much as 50 mμg were added per ml, no more than 3 mμg were bound. Pitney(2), using *Euglena gracilis*, reported average binding of 178 μμg/ml after adding 1,000 μμg, and noted that when more than 500 μμg were added per ml, most of the vitamin was unbound. Lear(3) reported similar findings with *E. gracilis*. Mollin(4) found that adding 20,000 μμg per ml serum resulted in binding of only 225 μμg. The above results with Co^{60} vit. B_{12} show a much higher binding capacity than any of the preceding reports. That this is not due to ionic

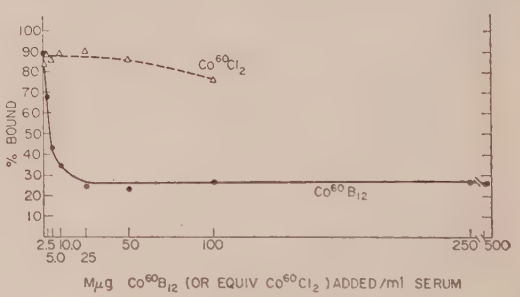

FIG. 1.

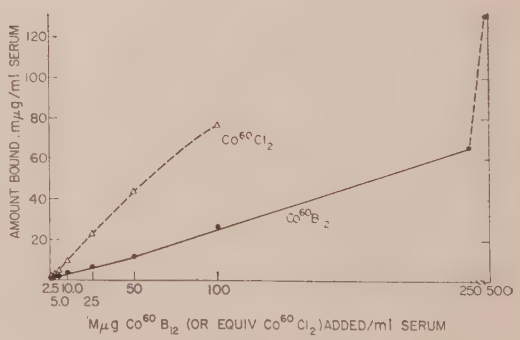

FIG. 2.

cobalt from possible breakdown of the vit. B_{12} molecule is suggested by the different binding capacity of serum for $Co^{60}Cl_2$.

Pitney(2) found vit. B_{12} bound to an alpha globulin *in vivo*. However, when the vitamin was added to serum *in vitro*, some travelled electrophoretically with beta globulin. This fraction of vitamin, unlike that bound to alpha globulin, was directly available to the test organism, and was consequently termed "free." The discrepancy in binding capacities assayed by the different methods of measurements may, therefore, occur because the amount of vit. B_{12} made available to a micro-organism by heating serum does not represent the total vitamin bound to the serum proteins. It is possible that though the vitamin is bound to an alpha globulin at usual body levels, this mechanism is quickly saturated and nonspecific binding to other serum protein fractions takes place at higher concentrations.

Summary. A method is presented for estimating binding capacity of serum for radioactive vit. B_{12}. This capacity was found to increase as the concentration of the vitamin was raised. "Bound" vit. B_{12} measured in this way is not synonymous with that considered bound because it is unavailable to certain microorganisms.

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Effect of Reserpine (Serpasil®)* on Oxygen Consumption of Euthyroid, Hypothyroid, and Hyperthyroid Guinea Pigs. (22889)

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Experimental observations regarding the influence of reserpine on thyroid function are contradictory. Although reserpine completely antagonized increase in oxygen consumption produced by exogenous thyroxine in rats(1), it produced neither significant change in metabolic rate(2) nor consistent alteration in 24-hour I^{131} uptake of thyroid gland of euthyroid rats(3). However, reserpine exhibits a significant direct antithyroid effect *in vitro*, an effect which consisted chiefly of inhibition of organic binding of I^{131}(4). Patients placed on prolonged reserpine therapy showed no change in thyroid status(3) or metabolic rate(5), and Ford, *et al.*(6) were unable to demonstrate any change in basal metabolic rate or I^{131} uptake of the thyroid gland in euthyroid patients after chronic treatment with total alkaloid extract of *Rauwolfia serpentina*. Moncke(7), however, found that chronic reserpine therapy over a period of months produced "normalization" of basal metabolic rate in a group of 15 hyperthyroid patients and was more effective than methyl thiouracil in combating cardiac arrythmias associated with the disease. When reserpine was used in combination with methyl thiouracil, smaller doses of both drugs were needed. Most studies have been concerned chiefly with the effect of reserpine in a single thyroid state.

To resolve the apparent dissimilarities in response to this alkaloid, experiments were conducted to compare its effects on oxygen consumption of hypothyroid, euthyroid, and hyperthyroid guinea pigs.

Materials and methods. A modification of

* Serpasil used was generously supplied by Ciba Pharmaceutical Products Inc., Summit, N. J.

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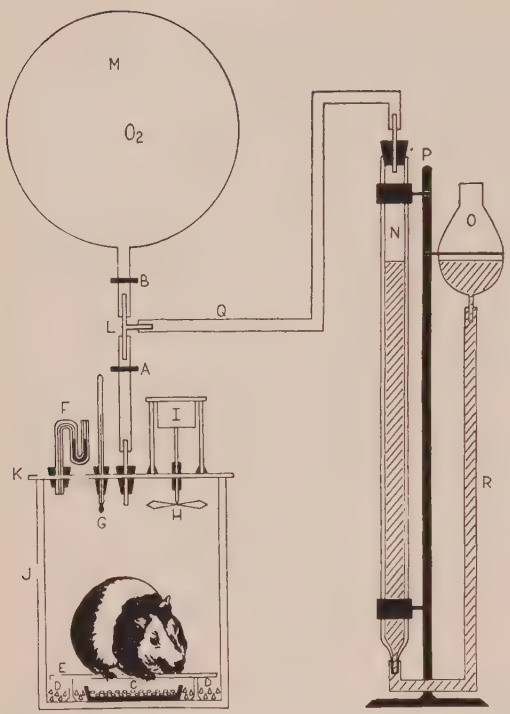

FIG. 1. Metabolimeter. A and B, clamps; C, anhydrous calcium chloride; D, soda lime; E, wire mesh platform; F, capillary tube manometer; G, thermometer; H, fan; I, motor; J, glass museum jar; K, brass plate cover; L, glass "T" joint; M, rubber bladder; N, calibrated burette; O, leveling bulb; P, ring stand; Q, rubber tubing; R, water.

Richards and Collison's(8) closed-chamber method was employed for determining oxygen consumption as a measurement of thyroid function (Fig. 1). The chamber, a circular glass museum jar, was 10 inches high and 8 inches diameter. A brass plate was used as cover. Water pump lubricant served to create an air-tight seal between cover and jar. A motor driven fan in upper part of chamber circulated air continuously. The fan shaft was sealed with lubricating oil. This permitted more rapid equilibration of gases and stabilization of temperature within the jar at 28 ± 0.5°C. Within the container the animal rested on wire mesh platform. Carbon dioxide and water vapor were absorbed by soda lime and anhydrous calcium chloride at bottom of jar. Reproducible readings were obtained if animals were allowed 25 minutes to come into thermal equilibrium. During equilibration and readings, the system was charged with 100% oxygen. Pressure changes within the jar were measured by a capillary tube manometer attached to cover. As oxygen was utilized, amounts sufficient to maintain atmospheric pressure within the system were delivered from a reservoir balloon. Oxygen consumed was measured at atmospheric pressure over water in a burette. When accustomed to the apparatus, the animals made only slight and infrequent movements during the experiments. Occasionally they became restless and an increase in consumption of oxygen followed. If movements lasted for some time, the increase persisted for a considerable time after movement ceased. For this reason, observations were made only during periods in which the animals manifested minimal activity. An average oxygen consumption for two 10 minute periods, which did not differ by more than ± 8%, was recorded. In the event of a greater difference, observations were extended until 2 consecutive periods were within limits. Since the observations of Cramer and M'Call (9) indicated that excess metabolism which followed eating of food by rats did not persist for more than 8 hours, our animals were starved for 12 hours prior to observations. The longer period was chosen to obviate variations due to the species of animal and the amount of food consumed. Thirty unselected guinea pigs, weighing 360 to 700 g, were divided into 3 equal groups, A (hypothyroid), B (hyperthyroid), and C (euthyroid), according to Fisher and Yates' tables of random numbers(10). Each animal occupied a separate cage in room in which the temperature was thermostatically maintained at 21°C. Ground Purina Rabbit Pellets, tap water, and supplemental cabbage were provided *ad libitum*. Drugs were thoroughly mixed in the ground food. Group A received 0.1% thiouracil for 5 weeks, Group B 0.1% desiccated thyroid§ for 3 weeks, while Group C served as controls. Thyroid and thiouracil medications were discontinued after signifi-

§ Desiccated thyroid USP Strong (0.3% I), was generously supplied by Parke, Davis and Co., Detroit, Mich.

TABLE I. Oxygen Consumption.

	Day	Liters oxygen/(kg body wt)$^{2/3}$ × 0.1/hr — Reserpine treated: Mean ± S.D.	Reserpine treated: Range	Solvent treated: Mean ± S.D.	Solvent treated: Range	% difference of means	Probability of diff. between means
Group A Hypothyroid	0	5.8 ± .58	5.5- 6.5	5.6 ± .56	4.5- 5.9	+ 3.6	>.2
	1	6.2 ± .49	5.7- 6.9	5.9 ± .73	5.0- 6.7	+ 5.1	"
	2	5.9 ± .36	5.5- 6.3	5.9 ± .70	5.0- 6.8	0	
	3	6.0 ± .52	5.8- 6.9	6.2 ± .58	5.3- 6.8	- 3.2	"
	5	5.6 ± .71	4.3- 6.1	5.9 ± .70	5.1- 6.7	- 5.1	"
	7	6.0 ± .58	5.5- 6.7	6.1 ± .75	5.5- 7.2	- 1.7	"
	9	6.2 ± .31	6.0- 6.8	6.7 ± .45	6.1- 6.9	- 7.5	"
	11	6.5 ± .48	5.8- 6.7	7.0 ± .33	6.5- 7.3	- 7.1	"
	12	6.9 ± .44	6.2- 7.3	7.1 ± .48	6.6- 7.6	- 2.8	"
Group B Hyperthyroid	0	9.5 ± .62	8.3-10.0	9.4 ± .58	8.9-10.3	+ 1.1	>.2
	1	8.1 ± .49	7.6- 8.9	8.9 ± .63	8.2- 9.8	- 9.0	<.1 but >.05
	1.17	7.2 ±1.29	4.9- 8.7	9.0 ± .70	7.9- 9.8	-20.0	<.05 but >.02
	2	7.2 ± .84	5.7- 8.2	8.8 ± .82	7.5-10.0	-18.2	*Idem*
	3	6.8 ± .47	6.0- 7.7	8.7 ± .64	8.0- 9.6	-21.8	<.01
	5	5.9 ± .81	5.4- 6.7	8.4 ± .71	7.3- 9.7	-29.8	"
	7	6.0 ±1.15	4.4- 7.3	7.7 ± .60	6.8- 8.5	-22.1	<.05 but >.02
	9	5.9 ± .36	5.4- 6.4	7.2 ± .20	7.0- 7.6	-18.1	<.01
	11	6.3 ± .36	5.8- 6.8	7.3 ± .46	6.9- 8.1	-13.7	"
	12	6.8 ± .22	6.6- 7.1	7.1 ± .32	6.7- 7.5	- 4.2	<.2 but >.1
Group C Euthyroid	0	7.2 ± .81	6.3- 8.0	7.2 ± .33	6.8- 7.8	0	
	1	6.7 ± .87	6.3- 7.8	7.3 ± .48	6.5- 7.8	- 8.2	<.1 but >.05
	2	5.8 ± .87	4.2- 6.6	7.3 ± .64	5.7- 8.1	-20.6	<.05 but >.02
	3	5.5 ± .68	5.1- 6.9	7.3 ± .20	7.1- 7.7	-24.6	<.01
	5	5.3 ± .99	4.6- 6.9	6.9 ± .45	6.3- 7.5	-23.2	<.05 but >.02
	7	5.9 ± .63	4.9- 6.9	7.3 ± .39	6.7- 7.8	-19.2	<.01
	9	6.4 ± .37	6.0- 7.0	7.4 ± .52	7.0- 8.3	-13.5	<.02 but >.01
	11	6.7 ± .47	5.9- 7.0	7.4 ± .66	7.8- 8.6	- 9.5	<.1 but >.05
	12	7.0 ± .19	6.7- 7.5	7.2 ± .50	6.7- 8.0	- 2.8	>.2

cant and stable degrees of hyperthyroidism and hypothyroidism were produced. At this time each group was divided into 2 subgroups composed of equal numbers of reserpine-treated and solvent-treated animals. Reserpine (Serpasil®) was injected intraperitoneally, 0.5 mg/kg on first day, followed by 0.35 mg/kg on second and third days, and 0.1 mg/kg on fourth day. Control animals received corresponding amounts of solvent vehicle in the same manner. Oxygen consumption was determined before starting reserpine therapy and at intervals of 4, 8, and 24 hours after each of the first 3 injections, and on days 5, 7, 9, 11, and 12. All values were corrected to standard temperature and pressure. Oxygen consumption was expressed as liters of oxygen/(kg body weight)$^{2/3}$ x 0.1/hour(11). The animals were autopsied at conclusion of experiments. Results were analyzed statistically for significance of the difference between group means by Student's "t" test(10).

Results. The data related to oxygen consumption have been summarized and their significance computed. Except in one instance, observations which were made at 4 and 8 hours, after each injection to detect acute effects of the drug, did not differ significantly from daily trends and were omitted for brevity (Table I). A graphic comparison of these same data (including 4 and 8 hour observations after each injection) is presented in Fig. 2.

Solvent-treated controls. Treatment with solvent had no effect on oxygen consumption of euthyroid guinea pigs, nor did it alter the gradual (12 day) return of oxygen consumption of hypo or hyperthyroid animals to euthyroid levels after cessation of thiouracil or thyroid treatment.

Hypothyroid animals. No significant differences in oxygen consumption between control and reserpine-treated hypothyroid animals were observed.

Euthyroid animals. Reserpine-treated euthyroid animals exhibited a significant decrease in oxygen consumption below corre-

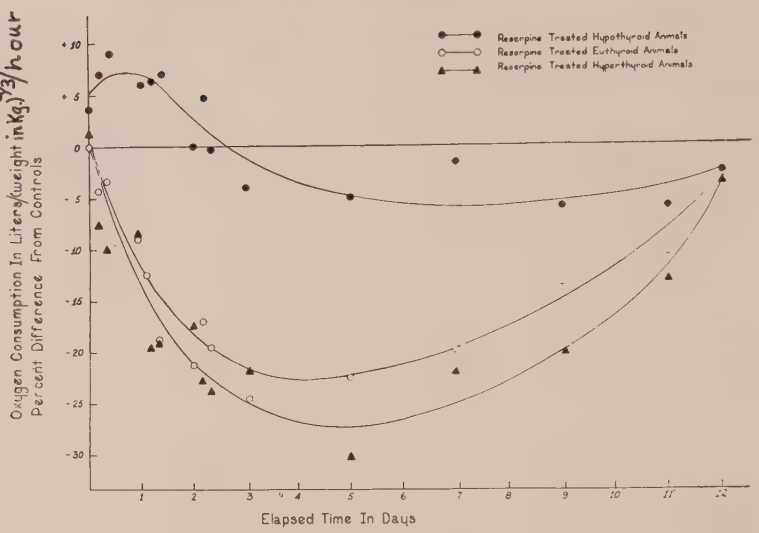

FIG. 2. Effect of reserpine treatment on oxygen consumption of hypothyroid, hyperthyroid and euthyroid guinea pigs.

$$\frac{\text{(Mean of reserpine-treated} - \text{mean of solvent-treated) } 100}{\text{Mean of solvent-treated}} = \% \text{ difference.}$$

sponding controls after 48 hours. The greatest effect occurred on the third day, at which time there was no difference between reserpine-treated euthyroid and the hypothyroid controls, which indicates that reserpine treatment had rendered the euthyroid subgroup hypothyroid. Oxygen consumption of reserpine-treated euthyroid subgroup was the same as that of controls by the twelfth day.

Hyperthyroid animals. A significant decrease in oxygen consumption of the reserpine-treated subgroup was observed after 28 hours, and the maximum effect occurred at 5 days. The reserpine-treated hyperthyroid animals were considered to be hypothyroid at this time, since there was no difference between these animals and the solvent-treated hypothyroid subgroup. Oxygen consumption gradually rose to euthyroid values at conclusion of the experiments.

There was a rapid decrease in weight of all 3 reserpine-treated subgroups (Table II) which was most marked in the hyperthyroid animals. The losses were maximum on the fourth day for all 3 subgroups and returned to within control values by the end of the experiment. No significant change in weights of thyroid, liver, spleen, kidney, adrenal, or lung was demonstrated, nor was there any gross evidence of infection.

Discussion. Oxygen consumption was reduced to hypothyroid levels in reserpine-treated hyperthyroid and euthyroid subgroups. No significant effect was obtained in the reserpine-treated hypothyroid subgroup. Since oxygen consumption was not reduced below hypothyroid levels in hyperthyroid and euthyroid animals, it appears that an antagonism for thyroid hormone might be the mechanism for reduction of oxygen consumption.

It seems likely that depression of oxygen consumption observed in reserpine-treated hyperthyroid animals probably was not due to an effect on the thyroid or pituitary since

TABLE II. Changes in Body Weights.

Group	% change in body wt at 4 days*		Probability of difference
	Solvent treated	Reserpine treated	
Hypothyroid	+4.3 ± 2.0	−11.5 ± 6.6	<.01
Euthyroid	+1.1 ± 3.7	−18.4 ± 12.7	<.02 but >.01
Hyperthyroid	−3.1 ± 5.0	−20.9 ± 4.9	<.01

* Includes mean and S.D.

suppression of activity of these glands by exogenous thyroid hormone has been well established(12,13). The failure of reserpine to antagonize the increase of oxygen consumption produced by 2,4 dinitrophenol(1) and depress oxygen consumption of hypothyroid animals makes a generalized depression of metabolism unlikely. These observations strengthen arguments, advanced by Kuschke and Gruner(1), for reserpine as a thyroxine antagonist. Similar responses of reserpine-treated euthyroid and hyperthyroid subgroups favor a similar mechanism of action operating in the 2 groups; however, a central hypothalamo-hypophyseal or a direct thyroid effect(4) are not eliminated. Nevertheless, it is important to consider that, although oxygen consumption and thyroid hormone activity are frequently associated, they are not necessarily related in any given instance.

The failure of reserpine to produce changes in metabolic rate(2,5) and I^{131} uptake by the thyroid(3) needs further clarification.

Changes in body weights which occurred in the reserpine-treated animals were probably due to decrease in food and water intake during drug therapy.

Summary. Oxygen consumption was reduced to hypothyroid levels in reserpine-treated euthyroid and hyperthyroid guinea pigs, but no significant changes were noted in the reserpine-treated hypothyroid animals. The evidence presented and discussed suggests that reserpine antagonizes the effect of thyroid hormone on oxygen consumption.

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Direct Exposure of Mice to Soils Known to Contain *Histoplasma capsulatum.* (22890)

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No report of natural infection of laboratory animals by direct exposure to soil known to contain *Histoplasma capsulatum* has appeared in the literature. However, animals have been successfully used to recover the fungus indirectly from soil(1) and from air samples(2) collected at point sources of human cases of histoplasmosis. Failure to isolate the etiological agent from soil and other material from sites of epidemics of histoplasmosis in humans has led to direct exposure of animals to these areas. In one reported epidemiologic study of an outbreak of histoplasmosis, a healthy dog, negative to histoplasmin skin test, was exposed to premises where humans and animals had become infected. This procedure resulted in disseminated histoplasmosis in the exposed animal(3).

It would be of interest to determine the effect of exposing animals directly on soils containing the mycelial phase of *H. capsulatum.* Through such studies the efficiency of the method to produce experimental histoplasmosis could be determined. Further evidence as to the natural method of contracting the disease might be obtained. In addition, a comparison of this method to that of usual technics for isolation of the fungus from soil could be made.

Methods. White Swiss mice, 4 to 5 weeks old, were exposed to 2 types of soil samples: one naturally contaminated; the other artificially inoculated with the mycelial phase of *H. capsulatum.* Naturally contaminated soil was collected 5 months previously from a farm where an epidemic of histoplasmosis had occurred. The fungus had been successfully isolated from this sample during routine epidemiologic studies. From the time of original collection, the sample was maintained at room temperature in the pasteboard carton in which it had been collected. During this period the soil had become quite dry. The artificial soil culture was prepared 16 months prior to its use by inoculating 100 ml of sterile soil contained in 250 ml Erlenmeyer flask, with a suspension of the mycelial phase of *H. capsulatum.* The soil culture was incubated at room temperature in a jar in which relative humidity of 100% was maintained. One week prior to the experiment, the flask was removed from the humidity jar and the soil allowed to dry. Approximately 100 ml of each type of soil was placed in the bottom of 2 clean, stainless steel mouse cages. Ten White Swiss mice were placed in each cage. Five control mice were placed in a third cage on clean sawdust. The 3 cages were placed in close proximity in a safety hood. Ultraviolet lights and blower to filter remained on. Mice were protected from exposure to ultraviolet rays by a layer of heavy wrapping paper between the light source and top of cages. After animals had been exposed to the soil for one week, they were transferred to clean cages and retained in the safety hood. Four weeks after removal from the soil the mice were sacrificed and autopsied. The liver, spleen and lungs of each mouse were cultured separately on Sabouraud's agar containing 20 units of penicillin and 40 units of streptomycin per ml. The plates were incubated at room temperature for one month, and observed periodically to determine the presence of *H. capsulatum.* By similar procedure, an attempt was made to determine the period of exposure necessary for mice to contract histoplasmosis. The naturally infected soil, proved positive for *H. capsulatum,* was obtained from a different epidemic site and maintained 10 months prior to its use. Identical artificially inoculated soil cultures were used. Twenty-one mice were exposed to each type of soil and 7 mice served as controls. At intervals of 1, 2, and 4 days, 7 mice were removed from each experimental soil and placed in clean cages. Four weeks after their removal from the soil, each group of mice was

TABLE I. Exposure of Mice for 7 Days to Soils Seeded with *Histoplasma capsulatum*.

Infected soil	No. days exposed	No. mice pos./No. autopsied	% positive	% tissue positive		
				Liver	Spleen	Lung
Natural	7	8/8	100	100	100	100
Artificial	7	9/9	100	100	100	100
Controls	*	0/5	0	0	0	0

* Not exposed.

sacrificed and autopsied. Tissues were cultured and observed in the same manner as the previous series.

Results. Table I presents the results of experimental histoplasmosis produced in mice exposed for 7 days to natural and artificially seeded soils with *H. capsulatum*. It is of interest that disseminated histoplasmosis occurred in all mice exposed to these soils for 7 days. The fungus was isolated from liver, spleen, and lungs of all experimental mice. No intestinal lesions or lymph node enlargements were observed. All tissues from control mice were negative for the fungus. Two animals from naturally contaminated and one from artificially seeded soils were destroyed by cannibalism during the 4 weeks following their removal from soils.

In the second series of experiments, periods of exposure to naturally and artificially seeded soil samples were varied. The results are summarized in Table II. All mice placed on artificially seeded soil developed disseminated disease after 1, 2 or 4 days of exposure. The number of colonies of *H. capsulatum* that developed on Sabouraud plates from various tissues, suggests little or no difference in degree of infection after 1, 2 or 4 days exposure on soils.

There was a difference in susceptibility of mice on contaminated soil in that one mouse exposed for one day and another exposed for 4 days did not yield positive cultures from any tissue. However, all 7 mice exposed for 2 days developed disseminated histoplasmosis. The fungus was isolated from liver and spleen of all 7 mice and from 6 of the 7 lungs cultured on Sabouraud's medium. The plates of lung tissue from one mouse were contaminated and overgrown within the first week of incubation. This contaminating fungus made it impossible to detect the presence or absence of *H. capsulatum* in lungs of this mouse. Examination of the intestines and lymph nodes of all animals revealed no gross pathology. However, no cultures were attempted from these tissues.

The 7 control mice were sacrificed at termination of experiment. Although these mice were in the same hood with the experimental mice for 5 weeks, no isolation of fungus was made from individual tissues.

Discussion. There is much speculation as to the significance of the extremely high histoplasmin skin test sensitivity among humans in certain localities of the United States. Many epidemiologists consider this sensitivity to result only from actual association with *H.*

TABLE II. Exposure of Mice for One, 2, and 4 Days to Soils Seeded with *Histoplasma capsulatum*.

Infected soil	No. days exposed	No. mice pos./No. autopsied	% positive	% tissue positive		
				Liver	Spleen	Lung
Natural	1	6/7	86	86	86	86
	2	7/7	100	100	100	86*
	4	6/7	86	86	86	86
Artificial	1	7/7	100	100	100	100
	2	7/7	100	100	100	100
	4	7/7	100	100	100	100
Controls	†	0/7	0	0	0	0

* Plates containing lung tissue from one mouse were contaminated.
† Not exposed.

capsulatum, although it is not certain that infection precedes sensitization. Environmental and epidemiological studies would suggest that the fungus is endemic in these localities and infection can result from contact with it in nature. Nevertheless, there is still no unanimity among epidemiologists as to the mode of infection in histoplasmosis. The results of many researchers point to the respiratory route as portal of entry of the etiological agent. In recent years the ever increasing number of epidemics in humans associated with dusty activities and of accidental infections among susceptible laboratory personnel would seem to support this hypothesis. The conditions under which infections have occurred among laboratory personnel leave little doubt that the disease was contracted by inhalation of the mycelial phase of the fungus from contaminated atmosphere. However, no controlled experiments involving humans have been reported to establish unequivocally the natural route of infection of histoplasmosis in man.

The experimental methods used simulated the natural environment of wild rodents and led to the development of histoplasmosis in the white mouse. Under conditions of these experiments, only the natural motion of mice produced dust aerosols. The large number of mice showing disseminated histoplasmosis with no apparent gross intestinal pathology is suggestive that the disease was contracted by inhalation of the fungus from dust aerosols. However, the experimental procedure did not completely preclude the possibility that other routes of infection existed, although these are unlikely(4).

The greater percentage of infections in mice exposed to artificially seeded soils was to be expected. Several previous determinations showed an average of 180,000 infectious units per ml of soil. Exposure for a period of several days to 100 ml of soil containing such a large number of viable infectious units certainly should have produced disease in susceptible animals.

In contrast to the favorable conditions under which artificially seeded soils were held, the 2 naturally contaminated soils, collected 5 and 10 months previously, were maintained at room temperature in the original pasteboard cartons. These soils had yielded *H. capsulatum* soon after their collection from the epidemic sites, using a modification of the flotation method followed by mouse inoculation. It was of interest to re-examine the results of these studies to ascertain the infectivity of soils at the time of the experiment. The naturally infected soil used in the first exposure study produced histoplasmosis in only 7 of 14 mice inoculated by the indirect method. It is significant that mice directly exposed for 7 days to this soil, after 5 months storage, resulted in 100% infectivity. All 7 mice inoculated indirectly with the second epidemic soil developed disseminated histoplasmosis. After storage for 10 months, this soil produced histoplasmosis in 90% of the animals directly exposed for 1, 2 and 4 days. No mice were exposed to this soil for 7 days. This factor, rather than the difference in storage time may account for the 90% infectivity by direct exposure of mice to this soil.

These preliminary studies indicate that the direct exposure method is more efficient than the indirect method of isolating *H. capsulatum* from contaminated soil. Since the mice do not have to combat bacteremia due to intraperitoneal inoculation by the indirect method, the mouse mortality rate is much lower by direct exposure. In addition, the direct method requires less time and equipment. However, further studies should be made to determine the efficiency of the direct method when only a few infectious units are present. Studies are now under investigation in an attempt to determine, on a large number of samples, the comparative efficiency of direct and indirect methods of isolating *H. capsulatum* from the same soil sample.

The universal infection acquired by mice under simulated natural conditions raises the question as to the fate of such naturally infected mice in nature. One wonders if such mice survive or die, since experimental evidence(4) suggests a high mortality after a rather long period of latency. On the other hand, no epidemiologic evidence of low mouse

populations or abnormal deaths has been reported in areas where the fungus has been known to exist in the soil. Further experiments on the ultimate fate of naturally infected mice are now in progress in an attempt to elucidate this problem.

Summary. 1. Susceptibility of white Swiss mice to infection with *H. capsulatum* by inhalation of dust aerosol was determined by direct exposure of animals to known positive soil samples. 2. Disseminated histoplasmosis occurred in a large percentage of mice. 3. Naturally contaminated soil samples held 5 and 10 months at room temperature remained highly infectious. 4. Artificially seeded soil cultures were 100% infective to the white mouse at exposures of 1, 2, 4, and 7 days. 5. These data add support to the hypothesis that man and animals become infected with *H. capsulatum* by the respiratory route. 6. The safety hood and associated safety procedures proved completely effective in preventing infection of the control mice although they were placed in close proximity to animals exposed to known positive soil samples.

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Human Amnion Cell Cultures; Susceptibility to Viruses and Use in Primary Virus Isolations.* (22891)

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Multiplication of the 3 types of poliomyelitis viruses in tissue cultures of human amnion cells has been reported by Zitcer, *et al.* (1). Because of abundance and availability of placental membranes, the studies reported here were initiated to determine the susceptibility of amnion cell cultures to other viruses and to investigate the possibility of large scale use of these cells for primary isolation of viral agents.

Materials and methods. Preparation of amnion cultures. Membranes were collected over a 14-hour period and placed in jar containing 200 ml sterile balanced salt solution to which penicillin and streptomycin had been added at a concentration of 100 units and 100 μg/ml respectively.† The jar containing the membranes was kept at room temperature until brought to the laboratory. After separation from the chorion, the amnion was washed in several changes of sterile balanced salt solution, cut into approximately 10 cm pieces and freed of blood clots by digestion in 0.25% trypsin solution for 30 to 45 minutes. The supernatant solution containing blood cells was then discarded and fresh trypsin solution added to the minced tissues. The cells were thereafter liberated by gentle agitation with a magnetic stirrer for 6 to 14 hours without decanting. This simplified the trypsinization process and cells obtained by this method did not appear to be damaged by the prolonged digestion. Cells were then centrifuged at 1,000 rpm for 10 minutes and washed twice in sterile balanced salt solution before being suspended at a 1/150 dilution in Eagle's synthetic medium supplemented with 10% horse serum. The yield of packed cells from one membrane varied from 0.5 to 1.5 ml. A small percentage of membranes yielded

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cells which failed to grow out in culture. Tube cultures were grown in stationary racks after being implanted with 0.5 ml of cell suspension. After 48 hours when cells had adhered to the glass, the cultures were fed with fresh growth medium. A confluent sheet of cells was usually obtained in 4 to 7 days, and cultures were thereafter maintained in Eagle's medium with 4% horse serum. All media contained 100 units penicillin, 100 μg streptomycin, and 50 units mycostatin/ml. *Viral susceptibility.* All the viruses used were high titer prototype strains employed by various investigators at the National Institutes of Health. Cultures were inoculated with maximal non-toxic concentrations of virus and followed for cytopathic changes. Fluids from virus-inoculated tubes which showed degenerative changes were harvested after maximum destruction had occurred and carried through 5 successive passages. Fluids from tubes showing no effects after observation for 18 to 21 days were also passed "blindly" through 5 successive passages after which fluids from the last passage were inoculated into other susceptible hosts in order to determine the presence or absence of virus. *Virus Isolation.* Throat swabs and washings collected in bacteriological broth from cases of febrile respiratory illnesses occurring in a general population group in the Washington, D.C. area during the winter of 1955-56 were tested in amnion cultures by inoculation of 0.2 ml of untreated material/tube and the tubes observed for 14 days for cytopathic changes. Adenoviruses were identified by the method described by Rowe, *et al.*(2) and poliovirus by the neutralization test.

Results. As previously described by Zitcer, *et al.*, cells derived from the amnion were epithelial-like and of two sizes, approximately 90% of the cultures being made up of the smaller cell type. Both cell types were indistinguishable when grown in culture (Fig. 1). Table I shows the results of tests on susceptibility of amnion cultures to the viruses tested.

Cultures inoculated with the 8 types of adenoviruses characteristically showed marked nuclear granularity, and infection

TABLE I. Viruses Tested in Human Amnion Cultures.

Virus	Source	Results*
Adenoviruses Types 1-8	HeLa cells	+
Coxsackie A1-8	Mouse	—
" A9	Monkey kidney cells	+
" B1, 3, 5	*Idem*	+
" B2†	"	±
" B4‡	"	—
Herpes simplex	HeLa cells	+
Influenza A, B	Monkey kidney cells	—
Dengue, Type 1	Mouse	—
Mumps	Chick embryo	—
Newcastle disease	*Idem*	—

* + = cytopathogenic through 5 passages. — = noncytopathogenic and negative for virus after 5 passages.
† One of 4 strains cytopathogenic.
‡ None of 4 " " .

was usually first noted in cells on the outer periphery of the cell sheet or on the lower end of the tubes (Fig. 2). Rounding of infected cells then followed, and the entire culture was usually degenerated by 4th to 8th day. This slow process of degeneration and the absence of clumped, rounded cells is in contrast with infection of this group of agents in HeLa cell cultures. In addition, acid production which is usually observed in HeLa cell cultures infected with adenoviruses(3) was not noted in adenovirus-infected amnion cultures grown and maintained in Eagle's medium.

Herpes simplex virus readily produced cytopathic changes in amnion cultures and with undiluted inoculum, foci of infected cells could be observed within 24 hours. Most of the cells were involved after 48 hours and formed clumps of rounded cells. (Fig. 3).

Of the group A Coxsackie viruses tested in amnion cultures, only type A9 produced cytopathic changes. Types 1, 3, and 5 of the Coxsackie B group readily caused cell damage, while only one of 4 B2 strains tested was capable of multiplication in amnion cells. None of 4 B4 Coxsackie strains showed any evidence of cytopathic changes or multiplication. Cell destruction caused by the Coxsackie viruses was usually rapid and definite within 24 to 48 hours after infection (Fig. 4).

Infectivity titers of representative strains of viruses after 5 passages in amnion cultures calculated by the Reed and Muench method were as follows: Type 3 adenovirus, $10^{3.8}$;

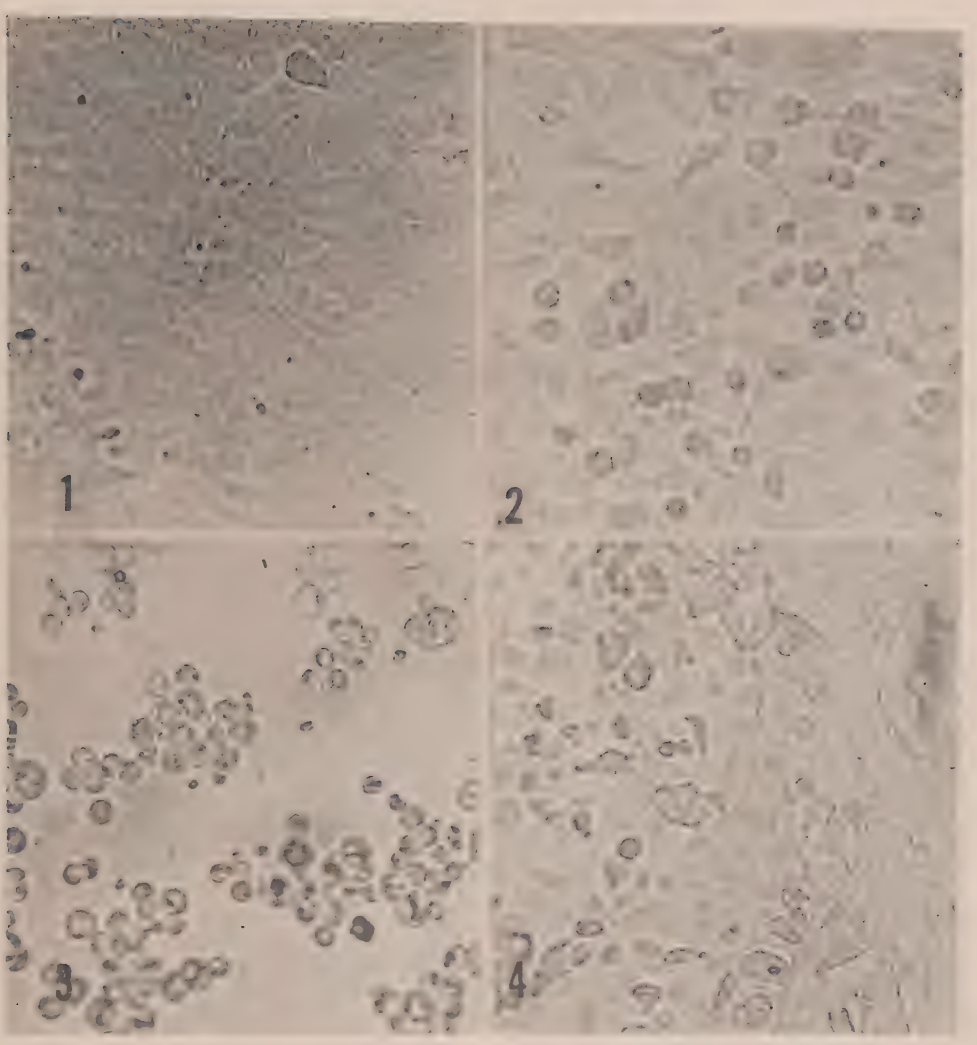

FIG. 1. 7-day-old culture of normal human amnion cells. Unstained. × 150.

FIG. 2. Early cytopathic changes in human amnion cell culture due to adenovirus type 3. Unstained. × 150.

FIG. 3. Cytopathic changes in human amnion cell culture 48 hr after inoculation with herpes simplex virus. Unstained. × 150.

FIG. 4. Early cytopathic changes in human amnion cell culture due to Coxsackie B1 virus. Unstained. × 150.

Coxsackie Bl, $10^{4.2}$; and herpes simplex virus, $10^{2.5}$. Titrations were made in amnion cultures and all tubes were observed for 14 days for cytopathic changes.

The high degree of susceptibility of amnion cells to the adenovirus group was further established in an experiment comparing the recovery of adenovirus types 1, 2, 3, and 5 from known positive throat specimens in amnion and HeLa cell cultures. The results of this experiment are shown in Table II. Of 16 specimens previously found positive for virus in HeLa cell cultures, 4 each of types 1, 2, 3, and 5 adenoviruses, virus was re-isolated in amnion cultures from all but one specimen, as compared with 12 isolations in HeLa cell cultures (obtained from the Microbiological Associates, Bethesda, Md.). Sixty unknown throat specimens taken from persons with febrile respiratory illnesses were also tested in both types of cultures, and adenoviruses were recovered from 5 in both amnion and

TABLE II. Comparative Isolations of Adenoviruses in Amnion and HeLa Cell Cultures.

Type of specimen	No.	No. isolations in Amnion	No. isolations in HeLa
Known positive	16	15	12
Unknown	60	5	5

HeLa cultures, all isolations being made from the same specimens.

Results of these experiments indicated that for isolation of adenoviruses, amnion cultures were as reliable as HeLa cultures. Moreover, the ease in maintaining amnion cultures made it seem that these cells would be of value for large scale use in routine screening for these and other viral agents. A total of 1,407 throat specimens, approximately half of which were taken from children, has been examined in amnion cultures and the results are shown in Table III. Forty isolations of adenovirus types 1, 2, 3, and 5 were made in amnion cultures. Except for one isolation of adenovirus type 5 from an adult, all strains isolated were from children whose ages ranged from 1 through 12. One type 1 poliovirus and 6 strains of herpes simplex virus were also recovered from the specimens tested.

For diagnostic purposes and for routine screening of materials for viral agents, amnion cultures may be a useful source of tissue culture cells. The range of viral susceptibility of amnion cells is very similar to that of the HeLa cell, and the results reported here are in accord with those of Weinstein, *et al.*(4).

TABLE III. Primary Virus Isolations in Human Amnion Cells.

No. specimens	Virus isolated	No.
1407	Adenovirus type 1	5
	Idem 2	13
	" 3	20
	" 5	2
	Poliovirus type 1	1
	Herpes simplex	6
	Total	47

Besides their susceptibility to a number of viruses, cultures of amnion cells can be readily prepared and easily maintained. In our hands, inoculated cultures can be maintained in a healthy state for periods as long as 3 to 4 weeks, with relatively infrequent medium changes. Spontaneous degeneration somewhat resembling viral effects was noted on 2 occasions, but no agent was recovered from culture fluids in amnion, HeLa, or monkey kidney cultures.

Preliminary experiments with cultures of human chorion have indicated that this tissue may also provide cultures suitable for viral studies. A mixed population of epithelial and fibroblastic cells usually is obtained from the chorion. The following viruses have been found to cause cytopathic changes in chorion cultures; adenoviruses (7 types), polioviruses types 1, 2, 3, herpes simplex virus, and the human salivary gland virus(5).

Summary. 1. Tissue cultures of human amnion cells were found to be susceptible to all adenoviruses tested, certain Coxsackie viruses, and herpes simplex virus. 2. Amnion cell cultures were employed in large scale routine testing for viral agents with recovery of 40 strains of adenoviruses types 1, 2, 3, and 5, one type 1 poliovirus, and 6 strains of herpes simplex virus.

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Manometric Studies on Guinea Pig Choline Oxidase.* (22892)

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It has been reported(1) that choline oxidase is absent from guinea pig liver. It was also reported later that the enzyme system is not found at all in the guinea pig and that the formation of methionine from choline is not demonstrable *in vitro* with guinea pig liver or kidney homogenates(2), using standard technics. However, Berg(3) has recently demonstrated, using C^{14} methyl-labeled choline, that both guinea pig liver slices and homogenates are able to methylate homocysteine to form methionine. This reaction was reported to be blocked under anaerobic conditions. From the latter observation, it appeared that choline oxidase must be present in the guinea pig, although its activity might be too low to be easily detected. We have reinvestigated this problem and wish to report our findings in the present paper.

Methods. Oxygen uptake studies using the Warburg manometric apparatus were undertaken to observe whether choline oxidase could be demonstrated in the guinea pig. The experimental animals were young adult guinea pig males fed a normal stock diet. After sacrificing, the liver, kidneys, and other organs were quickly removed and placed in ice. Homogenates (16.7%) were quickly prepared in 0.25 M sucrose using a Potter-Elvehjem homogenizer. The following concentration of buffers, at a pH of 7.3 unless stated otherwise, was used throughout the study: Krebs-Ringer phosphate (less calcium), 1.0 ml; and 0.039 M sodium potassium phosphate (equimolar in disodium phosphate and monopotassium phosphate), 0.5 ml. The filling of the Warburg vessels has been described elsewhere(4). The flasks were preincubated at 37°C for 1 hour before the addition of the substrate, choline chloride, was added from the side arm. The total fluid volume in each flask was brought to 3.7 ml with water. A control flask without substrate, but with water in its place, was used in each assay. Oxygen uptake was recorded for 180 minutes, after adding the substrate or water, at 10-minute intervals for the first 30 minutes and then at 30-minute intervals thereafter. Except for the studies concerning the effect of enzyme concentration on activity, the oxygen uptake was expressed as μl O_2 consumed due to added choline per hour per g tissue. The activity was calculated in every case from that portion of the "difference" (+ choline -- choline) curve which most nearly gave a straight line with maximum activity at least for 1 hour.

Results. The demonstration of the presence of choline oxidase and the optimum conditions for measuring the activity of the enzyme in guinea pig liver and kidney homogenates are presented in this section. Spleen, muscle, and intestine were also studied, but were found to contain no choline oxidase activity as measured in our system.

Preincubation effect. It was observed that endogenous oxidation in guinea pig homogenates was so high that choline oxidase activity is masked unless most of the endogenous oxygen uptake is first removed. For the first 60-90 minutes of the assay, manometer readings of the controls, representing endogenous oxidation, approached and sometimes exceeded manometer readings for the flasks containing added substrate. The difference in readings therefore would tend to lead one to believe that no activity was being measured. However, by preincubating the reaction mixtures for one hour before the addition of substrate, much of the endogenous respiration is removed. Lower manometer values are then observed for the control flasks and the difference values obtained represent a significant amount of oxidation taking place.

Effect of substrate concentration. As shown in Fig. 1, choline oxidase activity increased as the substrate concentration was

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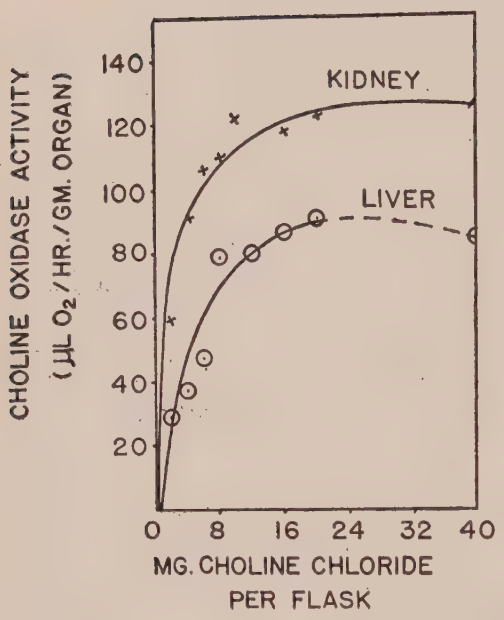

FIG. 1. Effect of substrate concentration on choline oxidase activities of guinea pig liver and kidney homogenates.

raised until about a 20 mg level of choline chloride per flask was reached. At first sight this might appear to be a rather high concentration of substrate but this feature is not unusual in enzyme systems. Possibly the inhibiting materials competing with the site of enzymatic activity in this system tend to cause a higher concentration of substrate to be required. Because of the low enzyme activity observed it cannot be stated with certainty whether a higher (40 mg/flask) level of choline chloride significantly inhibited the enzyme system, especially in the case of the liver homogenates. With rat liver a definite inhibition of the activity was observed for high substrate concentration(4).

Effect of pH. The buffer systems mentioned above were adjusted to various pH values and added to separate duplicate Warburg flasks (Set I). One ml of the appropriate homogenate was added to each flask. A separate set of flasks (Set II), similar to Set I was also prepared. The pH values were obtained by withdrawing the mixtures from the Warburg vessels of Set II after the initial 1-hour preincubation period plus 30 minutes after the addition of 20 mg of the substrate. The enzyme activity of Set I was recorded and plotted against the corresponding pH value. It was observed that a suitable pH optimum for both liver and kidney is 7.3.

Relation of enzyme concentration to activity. Enzyme activity was measured using volumes of 0.3, 0.5, 0.8, and 1.0 ml of 16.7% liver and kidney homogenates. Isotonic sucrose was added to equalize the enzyme volumes in the flasks. The substrate concentration was 20 mg of choline chloride and the pH of the buffers was 7.3. The enzyme activity was observed to be linear over the range of homogenate levels studied.

Location of choline oxidase in the cells of guinea pig liver and kidney. For these experiments 33% homogenates were used, since some activity would be expected to be lost during centrifugal fractionation. An aliquot of each homogenate was centrifuged at 25,000 g for 30 minutes in a refrigerated centrifuge. After separating the supernatant an equal quantity of isotonic sucrose was added to the insoluble residue and the mixture homogenized to resuspend the residue. The original homogenate, the resuspended residue, and the supernatant were then assayed for choline oxidase activity. The results indicate that the enzyme system, for both liver and kidney, is present in the insoluble fraction of the homogenate.

Identification of oxidation product in the kidney. A homogenate of 58 g of kidney in 290 ml of isotonic sucrose was preincubated with the two buffer systems in a 2-liter flask for 1 hour at 37°C. The volume ratio of the essential components of the reaction mixture in the Warburg vessels was maintained here. At the end of the preincubation period 174 ml of choline chloride equal to 6.96 g, stored at 37°C was added to the flask and the reaction mixture allowed to proceed under mechanical stirring for 6 hours. A control without added choline was run in the same manner. The reaction was terminated by precipitating the protein with trichloroacetic acid (T.C.A.). After removal of the T.C.A. with ether, the aqueous solution was concentrated under reduced pressure to a volume of

about 50 ml, and then treated with absolute alcohol to remove inorganic salts. After evaporating the alcohol at low temperature, the resulting aqueous solution was passed through a Dowex 50 column to remove the unused choline(5). Elution of the column with 1N HCl does not remove choline but does wash free other substances including betaine, a likely oxidation product of choline by the action of the enzyme. The hydrochloride elutant was concentrated to a small volume and then heated with charcoal to give a clear filtrate. A final concentrate of 5 ml was used in the identification of betaine by paper chromatography.

The literature reports various solvent systems† and detecting sprays for the paper chromatographic separation and detection of quaternary ammonium bases(6-11). Whatman No. 1 paper was used and the solvent allowed to ascend until a distance of 37 cm had been traversed. A control spot of betaine hydrochloride was applied on each strip. After air drying for 1 hour the detecting spray was applied. Betaine was identified only in the case where choline chloride had been added to the reaction mixture. Betaine aldehyde was not identified by an identical chromatographic process.

Discussion. In many cases, especially with liver homogenates, it was observed that choline oxidase activity was not measurable until a preincubation period of 1 hour and then did not commence until 60-90 minutes after the addition of substrate. This effect might be explained in several ways: 1. the lack of an adequate hydrogen-transport system to react simultaneously with choline dehydrogenase as well as with the endogenous-substrate oxidizing systems; 2. inhibition of certain endogenous oxidizing systems by choline chloride, which would give the impression that no choline oxidase was present until the substrates for those particular systems were used up; or 3. a lag in choline oxidation similar to that encountered with xanthine oxidase.

Although little or no activity was observed in the supernatant fluid of kidney and liver homogenates, it is possible that the enzyme system while insoluble, might require a cofactor. A likely site for this cofactor would be the supernatant fluid.

The identification of betaine as an oxidation product of choline should not rule out altogether the possibility of betaine aldehyde being present. The chromatographic methods employed for the identification of oxidation products of choline might not be sensitive enough to demonstrate the presence of betaine aldehyde.

Summary. 1. The enzyme system, choline oxidase, is present in the liver and kidneys of the guinea pig. Activity is low but measurable, as shown using standard manometric technics. 2. Optimum substrate concentration, pH, and assay conditions are presented. 3. The enzyme response *versus* enzyme concentration was found to be linear over the range tested. 4. The enzyme system in both liver and kidney of the guinea pig is insoluble as shown by centrifugal separation of the soluble and insoluble components of the homogenates in isotonic sucrose. 5. In kidney betaine was found to be an oxidation product by the action of the enzyme on choline.

† n-Butanol : Pyridine : Water(6;7,8)
3 : 1 : 1.5
n-Butanol : Ethanol : Acetic Acid : Water (9;7,10)
8 : 2 : 1 : 3
n-Butanol saturated with water(11;10)

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Effect of *H. pertussis* on Sensitivity of Mice to Cold Stress.* (22893)

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The injection of *Hemophilus pertussis* cells into mice of certain strains has been shown to produce pronounced changes in their sensitivity to histamine(1-3) and in their ability to become anaphylactically sensitive to various antigens(4-7). Adrenalectomized mice, like pertussis-treated mice, become highly susceptible to histamine(8) and to anaphylaxis(9). Adrenalectomized mice are more susceptible to stress than normal mice, and pertussis-treated mice have been found more sensitive to stresses such as x-rays(10) and low atmospheric pressures(11). These striking similarities between adrenalectomized and pertussis-treated mice have led to the inference that pertussis produces its effect mainly by interfering with adrenal function(11,12,13). This view is strengthened further by the fact that pertussis-treated mice can be protected by adrenal steroids (cortisone, hydrocortisone) against both histamine†(14,15) and anaphylaxis.† However, there is as yet no conclusive data that limits the explanation of the observed phenomena to variation in adrenal output. In fact, Gauthier *et al.*(16) have recently questioned the role of the adrenals in the development of histamine sensitivity in pertussis-treated mice.

The present report deals with the sensitivity of pertussis-treated and adrenalectomized mice to cold stress, and relates these observations to work previously reported.

Materials and methods. Swiss-Webster female mice, weighing from 14-16 g and purchased from Tumblebrook Farms, New York (T. F. mice), were used. The pertussis cells were prepared as previously described(3). Two billion cells contained in 0.2 ml were injected intraperitoneally into each mouse. Four days following administration of pertussis cells the mice were submitted to cold stress. For this purpose, each mouse was placed in an individual tin can with a wire top, and held in a cold room at 1-2°C for a period of 8 hours or longer. In mice requiring adrenalectomies, these were performed as previously described(8) and the mice were used not later than 24 hours after removal of the glands. When used, cortisone and hydrocortisone were suspended in saline and administered intraperitoneally 16 hours prior to cold stress. The experimental results were evaluated statistically by the methods of Wilcoxon(17) and Krushal and Wallis(18).

Results. Susceptibility of H. pertussis-treated mice and normal mice to cold stress. Twenty mice, injected with 2 billion *H. pertussis* cells, were exposed to cold stress 4 days following injection of cells. At the same time a group of 20 normal, untreated mice were placed in the cold to serve as controls. The results obtained conclusively demonstrated that pertussis-treated mice were more susceptible to cold stress than were normal mice. All but one of the treated mice died within 3 hours at 1-2°C while none of the normal mice died during this period. Even after 8 hours under conditions of this experiment 4 normal mice were still living. The difference between these 2 groups was shown to be highly significant ($P<0.001$). This experiment has been repeated several times with essentially the same results as may be seen in succeeding experiments (Table I, Groups I vs. IV).

Effect of adrenalectomy and sham adrenalectomy on cold stress. Adrenalectomized mice also become highly sensitive to cold stress. This is illustrated in the following experiment where in addition, the effect of sham adrenalectomy of normal and pertus-

* We wish to express our appreciation to Mr. J. L. Ciminera for statistical analysis of our experimental results, and to Dr. W. F. Verwey for his valuable suggestions and criticisms in preparing this manuscript.

† Unpublished observations, Munoz, J. and Schuchardt, L. F.; Munoz, J., and Baer, J.; Munoz, J., and Peck, H. M.

TABLE I. Effect of Adrenalectomy and Sham Adrenalectomy on Cold Stress.

Group No.	Treatment I	II	III	IV	V
Adrenalectomized	—*	+	—	—	—
Sham adrenalectomized	—	—	+	—	+
Pertussis	—	—	—	+	+
Time of death at 1-2°C (hr)	No. dead†				
1	0	0	0	0	0
2	0	0	0	1	2
3	0	2	1	7	5
4	0	2	0	2	3
5	0	3	1		
6	0	1	0		
7	3	2	1		
8	0		0		
>8	7		7		

* + = Received treatment. — = Did not receive treatment.

† Significance of difference: I *vs* II, $P < 0.001$; I *vs* III, not significant; I *vs* IV, $P < 0.001$; II *vs* IV, $P < 0.01$; IV *vs* V, not significant.

sis-treated mice was studied. Fifty mice, divided into groups of 10, were used. The mice of Group I were normal, untreated controls. Those of Group II were adrenalectomized, Group III were sham adrenalectomized, Group IV were treated with pertussis 4 days before cold stress, Group V were treated with pertussis 4 days before and sham operated the day before cold stress. The 5 groups were then submitted to cold stress. From the results given in Table I it can be seen that the adrenalectomized mice are more susceptible to cold than normal mice (Groups I and II), that the sham operated "normal" animals behave like the normal controls (Groups I and III), and that the sensitivity to cold of pertussis-treated mice is not affected by sham adrenalectomy (Groups IV and V). In addition, the results also show that pertussis-treated mice are more sensitive to cold than adrenalectomized mice (Groups II and IV).

Since the similarity of pertussis-treated mice to adrenalectomized mice is striking, it was of interest to investigate the effect of adrenal steroids on the cold sensitivity developed in mice treated with *H. pertussis*.

Effect of cortisone on pertussis-treated and adrenalectomized mice submitted to cold stress. Fifty mice, divided into groups of 10, were used. Two groups received pertussis, 2 groups were adrenalectomized, and 1 group was kept as normal controls. One group of each of the pertussis-treated and adrenalectomized mice received an intraperitoneal injection of 4 mg of cortisone per mouse 16 hours before cold stress, while the other groups received no treatment. The results given in Table II indicate that cortisone protected both pertussis-treated mice and adrenalectomized mice from cold stress, and that adrenalectomized mice appeared to have been protected better than pertussis-treated mice (Groups I and III).

Effect of hydrocortisone on cold sensitivity of pertussis-treated intact and adrenalectomized mice. It was of interest to know if the combination of pertussis treatment and adrenalectomy would increase the susceptibility of mice to cold stress more than either treatment alone. It was also important to know if mice receiving both treatments could be fully protected with adrenal steroids. In this experiment, hydrocortisone was used because it has been found to be more effective than cortisone. Twenty mice were used in each of 7 different groups. The mice in Group I received 2 billion *H. pertussis* cells 4 days

TABLE II. Effect of Cortisone on Susceptibility of *H. pertussis* Injected Mice and Adrenalectomized Mice to Cold Stress.

Group No.	Treatment I	II	III	IV	V
Pertussis inj.	+*	+	—	—	—
Adrenalectomized	—	—	+	+	—
mg cortisone/mouse	4	—	4	—	—
Time of death at 1-2°C (hr)	No. dead†				
1	0	0	0	0	0
2	0	0	0	0	0
3	0	4	0	1	1
4	1	4	0	1	1
5	2	2	0	0	2
6	1		1	4	0
7	2		1	1	1
8	1		1	0	0
>8	3		7	2	5

* + = Received treatment. — = Did not receive treatment.

† Significance of difference: I *vs* II, $P < 0.001$; I *vs* III, not significant; I *vs* V, not significant; II *vs* IV, $P = 0.006$; II *vs* V, $P < 0.01$; III *vs* IV, $P = 0.013$; III *vs* V, not significant; IV *vs* V, not significant.

TABLE III. Effect of Hydrocortisone on Sensitivity to Cold of Pertussis Injected and Adrenalectomized Mice.

Group No.	Treatment I	II	III	IV	V	VI	VII
Pertussis inj.	+*	+	—	—	+	+	—
Adrenalectomized	—	—	+	+	+	+	—
mg hydrocortisone per mouse	—	4	—	4	—	4	—
Time of death at 1-2°C (hr)	No. dead†						
1	0	0	1	0	1	0	0
2	2	0	6	0	17	5	2
3	10	4	3	1	1	6	0
4	5	6	2	3	1	4	2
5	3	3	5	1		2	0
6		2	3	3		1	1
7		1		1		0	0
8		0		0		0	3
>8		4		11		2	12

* + = Received treatment. — = Did not receive treatment.

† Significance of difference: I *vs* II, $P < 0.01$; I *vs* III, not significant; I *vs* V, $P < 0.001$; II *vs* IV, $P < 0.02$; II *vs* VII, $P < 0.02$; III *vs* V, $P < 0.01$; IV *vs* VII, not significant; VI *vs* VII, $P < 0.001$.

before cold stress, those of Group II received in addition to 2 billion *H. pertussis* cells 4 mg of hydrocortisone 16 hours before cold stress. Animals from Group III were adrenalectomized 24 hours before cold stress and Group IV were adrenalectomized 24 hours before and treated with 4 mg of hydrocortisone 16 hours before cold stress. The mice from Group V received 2 billion *H. pertussis* cells 4 days before and were adrenalectomized 24 hours before cold stress. Group VI was treated similarly to Group V but in addition each mouse received 4 mg of hydrocortisone 16 hours before being placed in the cold. The last group (Group VII) consisted of normal, untreated mice.

The results of this experiment are given in Table III. Here it can be seen that sensitivity to cold of pertussis-treated mice was, as previously noticed, slightly greater than that of adrenalectomized mice (Groups I and III) and that hydrocortisone, just as cortisone, protected adrenalectomized mice better than pertussis-treated mice (Groups II and IV). These observations confirm those made in the previous experiments. A highly important finding in this experiment is the fact that adrenalectomy of pertussis-treated mice renders them even more sensitive to cold stress than either treatment alone (Groups I, III and V), and that hydrocortisone, in the dose given, did not protect these mice completely (Groups VI and VII), while the same dose fully protected adrenalectomized mice (Groups IV and VII). Moreover, pertussis-treated mice are not fully protected by 4 mg of hydrocortisone (Groups II and VII). Since sham adrenalectomy does not change the sensitivity to cold of normal or pertussis-treated mice, the results indicate that pertussis may be interfering with functions other than that of the adrenal glands.

Effect of different amounts of hydrocortisone on sensitivity of pertussis-treated mice to cold stress. The observation that pertussis-treated mice were not fully protected from cold stress by hydrocortisone raised the question as to whether greater amounts of this compound could return the cold sensitivity of these mice to the normal level. To answer this question, the following experiment was performed. Seven groups of 10 mice each were used—Groups I, II, III, IV and V were given *H. pertussis* 4 days before challenge, and Groups VI and VII received no pertussis. Sixteen hours before cold stress, hydrocortisone was given to Groups II, III, IV, V, and VI; Group II received 1 mg/mouse; Group III received 2 mg; Group IV received 4 mg; Group V received 6 mg; and Group VI received 4 mg. Groups I and VII received no hydrocortisone.

The results given in Table IV show that there is a significant linear regression of mean reciprocal time on doses of hydrocortisone from 0 to 2 mg/mouse ($P < 0.01$). Larger doses of hydrocortisone up to 6 mg/mouse show no effect different than that given by 2 mg, indicating that 2 mg in this experiment gave the maximum protection to the pertussis-treated mice. Again, 2 mg or 4 mg of hydrocortisone failed to bring the pertussis-treated mice to their normal sensitivity since these 2 groups showed significantly lower survival times than the normal controls ($P < 0.05$). In this experiment, however, the difference between the normal group and that receiving 6 mg of hydrocortisone did not

TABLE IV. Susceptibility to Cold Stress of Pertussis Injected Mice Treated with Various Amounts of Hydrocortisone.

Group No.	Treatment I	II	III	IV	V	VI	VII
Pertussis inj.	+*	+	+	+	+	—	—
mg hydrocortisone per mouse	—	1	2	4	6	4	—
Time of death at 1-2°C (hr)	No. dead						
1	0	0	0	0	1	0	0
2	3	1	1	2	0	0	2
3	6	6	1	1	3	0	0
4	1	3	6	4	3	0	0
5			1	0	2	0	0
6			0	2	1	0	0
7			1	0	0	0	2
8				1	1	0	1
8.5					0	0	2
>8.5					1	10	3
Mean reciprocal time†	0.375	0.325	0.268	0.279	0.277	—	0.165

* + = Received treatment. — = Did not receive treatment.

† Significance of difference: There is a significant linear regression of mean reciprocal time on doses of hydrocortisone from 0 to 2 mg/mouse ($P < 0.01$). Larger doses of hydrocortisone up to 6 mg/mouse gave no significantly different results than 2 mg/mouse.

reach statistical significance. For this reason another experiment was performed using 20 mice/group and extending the observation period to 21 hours. In this second experiment 2 mice were placed in each tin can. For this reason longer survival times were observed as compared to those of the previous experiments. Four groups of mice were used. The mice in Groups I and II were injected with pertussis 4 days before cold stress while those of Groups III and IV were not. Sixteen hours before cold stress each mouse of Groups I and III received 6 mg of hydrocortisone intraperitoneally. The results demonstrated that pertussis-treated mice receiving 6 mg of hydrocortisone do not survive as long as the normal control mice. They also confirmed the finding of the previous experiment that hydrocortisone given to normal mice prolongs significantly their survival time at 1-2°C.

Discussion. Our observations as well as those reported by other workers are consistent with the view that mice treated with *H. pertussis* cells show signs of relative adrenal insufficiency. This is supported by the fact that pertussis-treated mice are more sensitive to various types of stress just as are adrenalectomized mice, and by the fact that pertussis-treated mice, as well as adrenalectomized mice, can be protected by cortisone and hydrocortisone. In spite of this apparently marked adrenal insufficiency, however, it has not been possible to demonstrate any adrenal pathology† or a significant change in the ascorbic acid content of the adrenal glands of mice that have been injected with pertussis.† These findings, while suggestive, nevertheless do not rule out conclusivly the possibility that pertussis may produce at least a temporary decrease in the activity of the adrenal glands. Although some of the results obtained here seem to warrant the assumption that pertussis interferes with adrenal function, it is difficult to explain all of the observations made by assuming that this is the only metabolic activity effected. All of these observations indicate that pertussis-treated mice are more sensitive to cold stress than would be expected from the complete elimination of adrenal gland function by the physical removal of the glands. Therefore, they suggest that the injection of pertussis cells while possibly affecting adrenal activity also produce an effect upon some other function, or functions, in the mouse that is concerned with protection against stress.

Accessory adrenal tissue, that takes over some of the adrenal functions after adrenalectomy, has been demonstrated in some mice (19). The presence of this tissue could possibly be considered to explain the differences observed between adrenalectomized and pertussis-treated mice with respect to cold stress. Such accessory adrenal tissue, however, is usually not highly developed and adrenal function activity can seldom be demonstrated 24 hours after adrenalectomy. For this reason it is improbable that the differences observed between pertussis-treated mice and adrenalectomized mice could be explained on the presence of accessory adrenal tissue. Moreover, this view would not offer an explanation as to why cortisone and hydrocortisone given in large

amounts did not protect completely pertussis-treated mice while fully protecting adrenalectomized mice, and why adrenalectomy of pertussis-treated mice further increased their sensitivity to cold.

Recently Gauthier, *et al.*(16) have strongly questioned the role played by the adrenals in pertussis-treated mice. Their conclusion that the adrenals are not involved in the histamine sensitization produced by pertussis finds support in our previous observations that adrenalectomy of certain strains of mice does not render them sensitive to histamine (8). The same resistant mice, however, when treated with pertussis become more sensitive to cold and to anaphylaxis.† These facts suggest that pertussis may indeed have some effect on the adrenal glands but that in addition it affects other functions in the mouse body.

Summary. Injection of 2 billion *H. pertussis* cells into T. F. female mice increases susceptibility of these mice to cold stress. 2. Susceptibility to cold of pertussis-treated mice is greater than that of adrenalectomized mice. 3. Adrenalectomy of pertussis-treated mice increases susceptibility to cold stress to a greater extent than either treatment alone. 4. Sham-operated animals whether normal or pertussis-treated are neither more resistant nor more sensitive to cold stress than similarly treated but unoperated animals. 5. Cortisone and hydrocortisone protect pertussis-treated mice from cold stress but to a lesser degree than they protect adrenalectomized mice. 6. Increasing the amount of hydrocortisone from 2 to 6 mg has little effect on increasing the protective effect of this drug. 7. Hydrocortisone given to normal mice increases their resistance to cold. 8. It is suggested that while pertussis possibly affects adrenal function, it must in addition affect other functions in the mouse which are also concerned with protection against stress.

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Comparative Plasma Levels of Mephenesin and its Carbamic Acid Ester. (22894)

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Mephenesin carbamate induces, in laboratory animals, profound skeletal muscle relaxation of considerably longer duration than that of its parent compound, mephenesin. Studies in the dog have indicated that mephenesin is oxidized to mephenesic acid or conjugated with glucuronic acid prior to urinary excretion. The fact that mephenesin carbamate is not oxidized to mephenesic acid in the dog may account for the relatively higher and more persistent plasma levels (with consequent prolonged pharmacologic activity) seen after its administration(1). The present study compares, in man, the plasma levels of mephenesin with those of its carbamic acid ester following oral administration at equal dosage of the respective drugs.

Methods. Control blood samples were obtained from 4 healthy male subjects, who then received single doses of 3 g of either mephenesin or its carbamic acid ester, orally with water. Citrated blood samples were obtained from each subject at ½, 1, 2 and 4 hours after administration of the drugs. The blood samples were spun down and plasma levels of the drugs were determined using a modification of the chromotropic acid method of Titus (2). One week later, in a cross-over experiment, the entire procedure was repeated, each subject receiving the alternate drug.

Results. Mephenesin initially attained higher plasma levels than did the carbamic acid ester. Its superiority persisted for only about 15 minutes. As shown in Fig. I, mephenesin carbamate was slower to reach its peak plasma level of approximately 13 μg per ml of plasma, but at 2 hours after administration of the drugs, mephenesin carbamate plasma levels exceeded those of mephenesin by 10 μg per ml, on the average. Four hours after administration of the drug, mephenesin was no longer detectable in the plasma, whereas after the same time interval, the average plasma concentration of mephenesin carbamate was 7 μg per ml. No free mephenesin was detected in the plasma of those subjects receiving mephenesin carbamate.

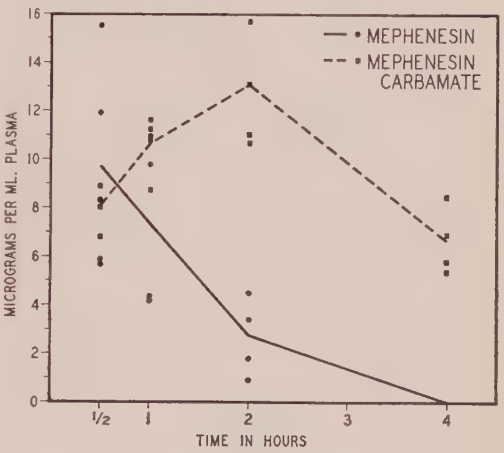

FIG. 1. Plasma levels of mephenesin and its carbamic acid ester during a 4-hr interval.

Summary. Mephenesin and its carbamic acid ester were orally administered to 4 normal males, the plasma levels of the respective drugs were determined at various intervals in each subject for 4 hours following administration of the drugs. Significantly higher and more persistent plasma levels were attained by mephenesin carbamate, as compared to those of mephenesin. No free mephenesin was found in the plasma of those subjects receiving mephenesin carbamate.

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Ionic Strength of Homogenizing Medium on Glutamic Dehydrogenase Activity of Tissues.*† (22895)

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Glutamic dehydrogenase activity in mammalian tissues has been studied by a number of investigators(1). No study, however, has been made as to relationship of activity with the medium used for homogenization of the tissues. In most instances, the buffer medium has been used but occasionally water has been employed. The low activity obtained while using a reported method(2) which prepared the tissue homogenate in phosphate buffer prompted this study.

Methods. Adult male mice of Swiss strain, (National Laboratories, Creve Coeur, Mo.) and rats (Sprague-Dawley) were used. Tissues were homogenized in all-glass apparatus and diluted to 1% for liver and 2% for kidney. The glutamic dehydrogenase activity was determined at 37° by DPNH oxidation method of Olson and Anfinsen(2) with a Cary Spectrophotometer.‡ The DPNH preparation was 91% as compared to 60% used by Olson and Anfinsen(2). Therefore, 50% more DPNH was used. This amount of DPNH gave a linear oxidation with time while higher and lower amounts were not completely satisfactory. The DPNH was prepared just before use from pure Pabst DPN by the method of Green and Dewan(3). The average of conversions was 91 ± .3% as determined by extinction coefficients at 260 and 340 mμ. Solutions were prepared in distilled water which had been passed through a deionizer column (Deiminizer, A. S. Aloe Co.).

Results. The relative glutamic dehydrogenase activity of mouse liver and kidney decreased rather slowly until an ionic strength of about 3 x 10^{-3} was used in the homogenizing medium and then decreased very rapidly (Fig. 1). The results were not due to any specific ion because identical results were obtained with the 3 different salts. The same phenomenon was observed in the preparation of rat liver and kidney homogenates with phosphate buffer (Fig. 2).

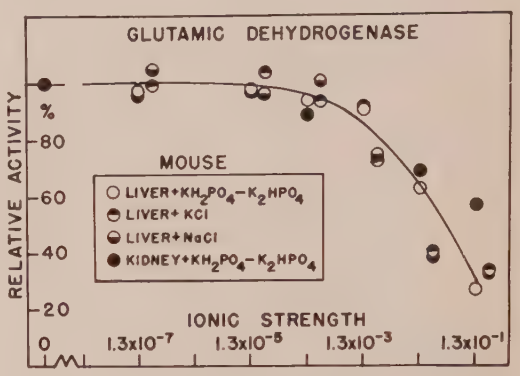

FIG. 1. Relationship of glutamic dehydrogenase activity of mouse liver and kidney to ionic strength of homogenization medium.

The decrease in activity was not due to presence of ions in the assay medium, for addition of KCl at an ionic strength of 3 x 10^{-1} after homogenization in water did not decrease the activity. Addition of much higher concentrations of several ions to the assay medium has been reported(2) to decrease en-

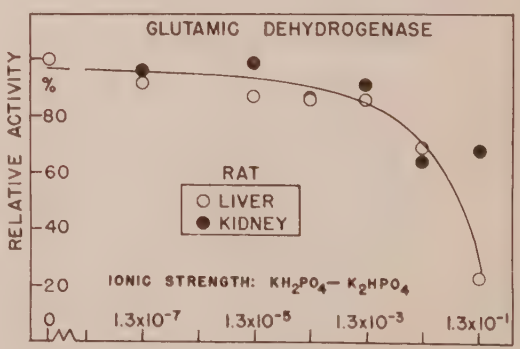

FIG. 2. Relationship of glutamic dehydrogenase activity of rat liver and kidney to ionic strength of phosphate buffer, pH 7.4, used for homogenization.

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† James Rhoads assisted in parts of this study.

‡ The Cary Spectrophotometer was provided by the Amer. Cancer Soc.

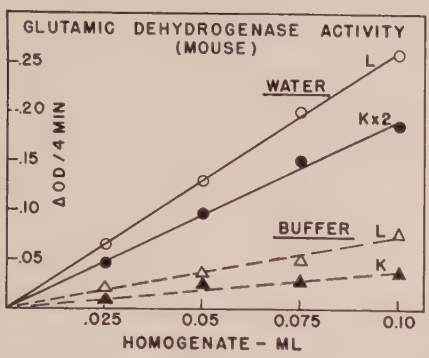

FIG. 3. Relationship of glutamic dehydrogenase activity of mouse liver and kidney to amount of homogenate used in the assay. The homogenates were: liver 1% and kidney 2%. Twice the amount of kidney homogenate prepared in water was used.

zyme activity.

Activity of glutamic dehydrogenase of liver and kidney homogenized in water was proportional to the amount of homogenate used in the assay (Fig. 3).

Discussion. The increase in glutamic dehydrogenase activity when the homogenates are prepared in water is probably due to a greater release of the enzyme from the mitochondria. It has been reported that the mitochondria swell in ionic solutions and burst in water(4).

Summary. Glutamic-dehydrogenase activity of liver and kidney of the mouse and rat was greater when the tissues were homogenized in water than in .05 M phosphate buffer, or NaCl and KCl of equivalent ionic strength. The decrease in activity could be varied by variations in ionic strength of the medium.

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Mode of Action of Potato Apyrase.* (22896)

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In a previous paper(1), we reported on an enzyme preparation from potato which selectively catalyzes hydrolysis of acid-labile phosphates of adenosinetriphosphate (ATP) and shows only negligible activity toward adenosine-5-phosphate and inorganic pyrophosphate. Several laboratories(2,3,4) have since verified our results bearing on the selectivity of action and, recently, Whittam, Bartley and Weber(4) confirmed our observation that at low temperatures (0°-7°), the enzyme preparation catalyzes hydrolysis of ATP only to adenosinediphosphate (ADP) and inorganic phosphate, while at higher temperatures both labile phosphates of ATP are removed.

Despite frequent practical uses to which the preparation has been put(2,4,5), its mode of action remains obscure. Conceivably, the preparation may consist of a single active protein showing greatly different activities toward ATP and ADP. However, we have not been able to demonstrate any evidence of competition between the two substrates. It is also possible that the preparation may consist of a mixture of enzymes whose ratio remains constant during various fractionation procedures we have employed. Either a mixture of an ATPase and an adenylate kinase, or of an ATPase and an ADPase would explain the enzyme activities observed. The observations(6) that plant materials contain adeny-

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late kinase prompted this study of the possible presence of this enzyme in the potato preparation.

Materials and methods. ATP, as the barium salt, was isolated from rabbit muscle according to modification of method of Dounce *et al.*(7). ADP was prepared by modification of the procedure of Bailey(8). Yeast hexokinase was purified to the 3a stage as described by Berger *et al.*(9). The potato enzyme was prepared according to Lee and Eiler(1). Inorganic phosphate was determined by the method of Fiske and Subbarow (10). The labile phosphate of either ATP or ADP was measured by increase in inorganic phosphate due to hydrolysis of either substance in 1 N sulfuric acid at 100° for 10 minutes. The reaction mixture and conditions for the enzymic reactions are given in Table I. It should be noted that the potato enzyme can be activated with either calcium or magnesium serving as the divalent cation. Hexokinase was used to test activity of adenylate kinase. With ADP as substrate, the presence of adenylate kinase in the preparation would provide ATP, which, in the presence of added hexokinase and glucose, would account for loss in labile phosphate not accompanied by an increase in inorganic phosphate. Such a loss in labile phosphate is assumed to represent hexose phosphate in these experiments. In this test, hexokinase must compete with ATPase activity of the preparation for the ATP that is to be released if adenylate kinase is present. Further both enzymes will be competing for low concentrations of ATP since both enzymes are highly active in comparison to the ADP splitting activity of the preparation. It can be shown that factors influencing competition between two enzymes for a single substrate are related as follows: $\frac{v''}{v'} = \frac{V'' \cdot [Km' + (S)]}{V' \cdot [Km'' + (S)]}$ where the v's are the respective velocities at a given concentration of substrate (S), the V's are the respective velocities at high substrate concentration; and, the Km's, assumed to approximate true dissociation constants for the respective enzyme-substrate complexes, are the Michaelis constants. At concentrations of substrate less than the Km's, as expected to be obtained in these studies, the above relation reduces to the approximation: $\frac{v''}{v'} = \frac{V''}{V'} \cdot \frac{Km'}{Km''}$. To adjust the concentrations of the competing enzymes, hexokinase and the ATP splitting enzyme of the potato preparation, so that they will be competitive at low concentrations of ATP, it is desirable that the Km's relative to ATP for both enzymes be known. The Km for yeast hexokinase has been reported by Slein *et al.*(11). We determined the Km for the ATPase activity of the potato preparation, using the conditions given in Table I, over a 100-fold range in concentration of ATP.

TABLE I. Hydrolysis of ATP and ADP by Potato Apyrase and Hexokinase. Each tube contained: $MgCl_2$ (0.006 M); succinate buffer, pH 6.8 (0.02 M); glucose (0.05 M); ATP (395 μg acid-labile-P) or ADP (350 μg acid-labile-P) and enzymes* as indicated in the table. Final vol was 2.8 ml. Incubation time for ATP as substrate was 5 min. at 30°C, and for ADP as substrate was 30 min. at 30°C.

	After incubation†		
	Inorg. P	Acid-labile-P	Hexophosphate-P
ATP as substrate			
No enzyme	0	395	
Potato enzyme	180	218	
Hexokinase	0	251	144
Potato enz. & hexokinase	102	197	96
ADP as substrate			
No enzyme	0	347	
Potato enzyme	92	258	
Hexokinase	0	350	
Potato enz. & hexokinase	105	245	

* See text for enzyme concentrations.
† All quantities in μg.

Results. From the data presented in Fig. 1, calculations yielded a Km of 1.4 x 10^{-4} M for the ATPase activity of the preparation. This is very close to the value of 9.5 x 10^{-5} M reported for yeast hexokinase(11). According to the approximate equation given above, the two enzymes would be competitive for low concentrations of ATP if their concentrations were adjusted so that they would be competitive at high concentration of ATP, since their Km's are nearly equal. In obtaining the data presented in Table I with ATP

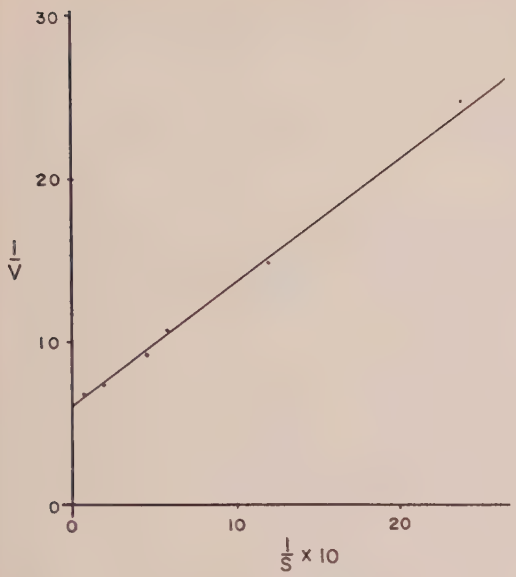

FIG. 1. Lineweaver-Burk plot on effect of ATP conc. on rate of hydrolysis by potato enzyme. Conditions as in Table I. Ordinate is reciprocal of rate of hydrolysis of ATP (in millimoles/l) at 30° during 5 min., and abscissa is reciprocal of conc. of ATP (in millimoles/l).

as substrate, the concentrations of hexokinase and the ATP splitting activity of the potato preparation were adjusted so that their respective activities were approximately equal when the reaction mixture contained 395 μg acid-labile phosphate (ATP) per 2.8 ml. When tested separately, or in competition with each other, the two enzymes showed approximately equal activity, as judged by the increase in inorganic phosphate and loss of labile phosphate not giving rise to inorganic phosphate (hexose phosphate). The same concentrations of the two enzymes were then tested separately and in competition with each other with ADP serving as the substrate. The data in Table I with ADP as substrate show, both in the presence and in the absence of hexokinase, that inorganic phosphate accounted completely for the decrease in acid-labile phosphate. Clearly, ATP is not an intermediate in the hydrolysis of ADP and adenylate kinase is not present in the preparation.

Since ADP in moderate to high concentrations does not inhibit the rate of hydrolysis of ATP at pH 6.8,‡ it seems probable that at least two sites of enzymic activity are present in the preparation. Presumably, the second site may be described as having ADPase activity. The two enzymic functions have different pH optima. In the presence of 0.033 M succinate buffer, the ATP splitting activity shows a sharp optimum at pH 6.4-6.5, while in the same buffer the ADP splitting activity shows an equally sharp optimum at pH 4.4.‡

Summary. With the use of hexokinase, it has been possible to show that the adenylpyrophosphatase from potato does not contain adenylate kinase. The lack of competitive inhibition of ATP splitting by ADP suggests two sites of enzymic activity, one acting on ATP, the other on ADP.

‡ Unpublished data.

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Adenine and Adenosine Deaminase Activity of Rat Mammary Gland Homogenates Through Pregnancy and Lactation.* (22897)

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Various studies of mammary gland growth and milk secretion which have been carried out in this laboratory in the last few years have indicated the importance of nucleic acids, pentose nucleic acid and deoxypentose nucleic acid. Kirkham and Turner(1,2) have reported the changes in PNA and DNA content which occur in the rat mammary gland through pregnancy and lactation and in the gland stimulated to growth with estrogen and progesterone. These changes were described by Yamamoto and Turner(3) for the rabbit mammary gland stimulated to growth with various levels of estrogen and progesterone. These studies indicate an increase in total DNA as gland growth occurs and an increase in PNA content of the gland throughout pregnancy and lactation which probably corresponds roughly to the amount of synthesis occurring. Williams and Turner(4) have reported studies in the rabbit which implicate PNA in the action of lactogenic hormone on the mammary gland. Morgan *et al.*(5) reported the presence of adenine deaminase and xanthine oxidase in cow's milk, which would suggest the possible presence of these enzymes of purine metabolism in mammary tissue. Adenosine deaminase, another of the enzymes of purine metabolism, was reported present in many mammalian tissues by Conway and Cooke(6).

The presence of these enzymes in mammary gland tissue, which is suggested by the work cited, and their activity during gland growth and milk secretion might be indicative of various biochemical and physiological changes during growth and milk synthesis. The presence or absence of these enzymes would suggest the presence or absence of certain pathways of metabolism for adenine and its derivatives. Further, changes in level of enzyme activity if correlated with physiological processes being studied might indicate more completely the nature of these changes at the cellular level.

Methods and materials. Female albino rats weighing approximately 150 g were used at various stages of mammary gland development. These physiological states were nonpregnant, pregnant, lactating, and involuting animal. In all cases the animal was sacrificed, the mammary tissue rapidly dissected free, with as little connective tissue as possible, and chilled on ice. The mammary tissue was then homogenized for 3 minutes in Waring blendor, in the cold, with 9 volumes of isotonic NaCl. The homogenate was then strained through 4 layers of cheese cloth. The strained homogenate was assayed immediately for its deaminase activity toward adenine and adenosine. The *assay* of the rate of deamination of adenine and adenosine was carried out using manometric technic of Warburg as described by Zittle(7) for determination of liberated ammonia. The *reaction flask* contents for all determinations were as follows: 1.4 ml of 1.15 M $NaHCO_3$ gassed with CO_2, 1 ml of mammary gland homogenate, and 5 mg of substrate. The gas phase was 100% CO_2 and temperature 38°C. The final volume was 3.4 ml at pH 7. The Warburg flask contents were assayed for DNA content using the *p*-nitrophenyl hydrazine method of Webb and Levy(8) because of its high sensitivity.

Results. Under conditions of this study there was apparently no deamination of adenine by rat mammary gland homogenates. This was found to be the case at all stages of pregnancy, lactation and involution.

On the other hand, these mammary gland homogenates showed a marked ability to deaminate adenosine in all physiological states studied (Fig. 1).

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† Public Health Service Research Fellow of Natl. Cancer Institute.

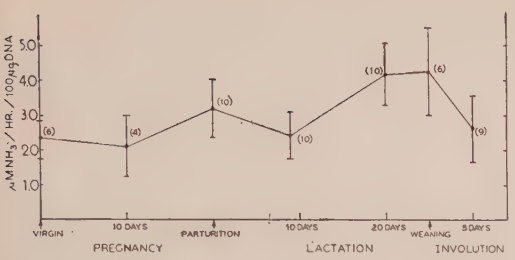

FIG. 1. Adenosine deaminase activity during pregnancy, lactation and involution in the rat mammary gland.

The changes which were noted in ability to deaminate adenosine under different physiological conditions studied are of interest. An apparent increase in adenosine deaminase activity was noted in the last third of pregnancy. However, this was not statistically significant. During early lactation deaminase activity was approximately the same as found in the gland of virgin and pregnant animals. During the last half of lactation there was a marked rise in adenosine deaminase activity from a mean of 2.41 μM/hour/100 μg DNA in early lactation to 4.17 μM/hour/100 μg DNA in late lactation. This increase was found to be highly significant ($P < .01$).

It appears, then, that the mammary gland of the rat contains no adenine deaminase and that adenosine deaminase is present and increases in the latter half of lactation.

Discussion. The lack of adenine deaminase activity in these rat mammary gland homogenates is in accord with previous studies of other tissues by Conway and Cooke(9), Richert and Westerfeld(10), and Block and Johnson(11). This would appear to indicate that the mammary gland does not catabolize adenine via deamination to hypoxanthine. Christman(12) and Brown(13) have reviewed the studies of nucleic acid precursors which indicate that in the rat adenine is utilized as a precursor of nucleic acid adenine and guanine. The apparent lack of adenine deamination may be related to its utilization in the pronounced synthesis of nucleic acids in the mammary gland which was demonstrated by the work of Kirkham and Turner(1,2) and Yamamoto and Turner(3) to occur during pregnancy and lactation.

From these studies it appears that adenosine is deaminated by mammary gland throughout pregnancy and lactation. This may indicate that adenosine as such is not utilized in the synthesis of the mammary gland nucleic acids. The presence of adenosine deaminase and the apparent lack of adenine deaminase would seem to indicate a pathway of catabolism for the adenine compounds which proceeds via deamination to inosine.

The rise in adenosine deaminase activity noted in the latter stages of lactation may be indicative of the onset of involution which would involve increased nucleic acid breakdown. This, however, may not be the answer as adenosine deaminase activity was not found to be increased in the involuting mammary gland.

Summary. Adenine deaminase activity and adenosine deaminase activity of rat mammary gland homogenates were studied through pregnancy, lactation and involution. Adenine deaminase activity was not apparent in the mammary gland. Adenosine deaminase activity was present throughout pregnancy and lactation, showing maximum activity in the latter stages of lactation.

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Evaluation of Synthetic Substrate Methods for Clinical Determination of Fibrinolysis. (22898)

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During the course of a clinical study on fibrinolysis, the question arose as to desirable methods for the quantitative estimation of fibrinolysis. The method of fibrin determination(1) had been in use at this laboratory. It was hoped that a more rapid method could be found since this method requires a fairly long incubation period. An investigation was begun on the utilization of synthetic substrate methods which have been reported to measure the activity of purified plasmin(2,3). Although the number of determinations in this study is not large, it is felt that it is sufficient to demonstrate the clinical inapplicability of the synthetic substrate methods as a measure of spontaneous fibrinolysis in plasma.

Materials and methods. Citrated venous blood was obtained from healthy personnel of this laboratory and from patients in whom marked fibrinolysis was induced by intravenous injection of pyrogens.* The cells were separated from the plasma immediately after drawing by centrifugation at 2000 rpm for 10 minutes. An aliquot of the plasma was diluted 1:4 for the lysine ethyl ester and tosyl arginine methyl ester assays. These assays were performed as described by Troll and Sherry(2,3), measuring both the spontaneous (SP) and streptokinase† (SK)-activated activity at 0, 40 and 60 minutes of incubation. The results are expressed as mean increase in micromoles of acid liberated. A second aliquot of plasma, diluted 1:25 in a 0.1 M $CaCl_2$ solution, was used for the determination of fibrin following a 45-minute and 24-hour incubation. The amount of fibrin was estimated by its tyrosine content(1).

Results. Blood was drawn at random from 8 normal individuals, 4 of whom had blood drawn on 4 successive days. These bloods were run only in the ester assays. Extreme variation was shown with these assays, both from individual to individual (Table I) and in one individual from day to day (Table II). Only the data for the 60-minute incubations are shown since a statistical analysis showed a similar variation in the 40- and 60-minute values.

It was thought that possibly diet might be a factor in this variation. Therefore, fasting samples of blood were taken from 8 normal individuals, 5 of whom had been in the random group. On these 5 normals, blood was drawn on the same day one hour after coffee and 1½ hours after lunch. The series was repeated for 3 days. The ester assays and the fibrin method were performed on all the plasma samples. The results from the fasting samples showed as extreme a variation in the ester assays as in the random group. There were variations in the enzyme activities following coffee and lunch but no consistent trends (Tables I and II). Comparing the 24-hour with the 45-minute incubations in the fibrin method, a mean increase of 2.3% of fibrin in the fasting group, an increase of 1.9% in the post coffee group, and a decrease of 10.9% in the post lunch group was observed. No lysis was shown in any of the normals.

The plasmas from the 6 patients treated with pyrogens showed complete spontaneous lysis in 30 to 120 minutes with the fibrin method but both the spontaneous and SK-activated specimens fell within the normal range in the ester assays (Table I). Pretreatment bloods were drawn on 3 of the patients, one of which lysed within 45 minutes and can

* These samples were kindly donated by Dr. K. N. von Kaulla, Univ. of Colorado, and analyzed one hour after drawing. Pyrogen administered was 300 μg of preparation No. 1083, Wander Co., Chicago, Ill.

† Varidase (10,000 units/cc.), Lederle Laboratories Division, Am. Cyanamid Co., N. Y. L-Lysine Ethylester Dihydrochloride and p-Toluene Sulfonyl L-Arginine Methylester Hydrochloride, Mann Research Laboratories, N. Y.

TABLE I. Group Comparison of Results from Ester Assays. (Expressed as mean increase μmoles of acid liberated per 60 min. incubation.)

	Mean		Range (2σ)		% in normal range	
	SK	SP	SK	SP	SK	SP
Lysine:						
Normals:						
Random (18)*	.59	.09	.37-.81	.0 -.19	100	100
Fasting (14)	.69	.08	.33-1.05	.0 -.34	100	92.9
Post coffee (11)	.62	.12	.16-1.08	.0 -.46	100	100
" lunch (6)	.71	.15	.35-1.07	.0 -.35	100	100
Patients (fibrinolytic) (7)	.44	.05	.20- .68	.0 -.13	100	100
Tosyl arginine:						
Normals:						
Random (19)	.55	.19	.39- .71	.09-.29	100	94.7
Fasting (15)	.66	.20	.32-1.00	.0 -.44	100	100
Post coffee (11)	.60	.17	.24- .96	.0 -.55	100	100
" lunch (8)	.57	.15	.19- .95	.0 -.31	87.5	100
Patients (fibrinolytic) (7)	.67	.25	.49- .85	.13-.37	100	85.7

* No. of plasma samples.

be included with the lytic plasmas. The other 2 controls showed no lysis with the fibrin method while the ester assay values were similar to those of the respective lytic plasmas. Although only the spontaneous enzyme activities can be compared with the fibrin method, it is of interest to note that there is also no difference between the activated lytic and non-lytic plasmas.

A longer period of incubation of 120 and 180 minutes was done with the ester assays on the lytic blood and normals. The increased period of time brought out no difference between the values for the lytic and non-lytic. Also, undiluted samples were used in the ester assays, again showing no difference between the two.

TABLE II. Individual Variations with Ester Assays. (Values represent mean increase in μmoles of acid liberated in 60 min. incubation.)

	Fasting		Post coffee		Post lunch		Random	
	SK	SP	SK	SP	SK	SP	SK	SP
Lysine:								
LM	.98	.016	.83	.15	.88	.14	.76	.15
JC	.58	.045	.53	.14	.56	.14	.47	.06
RS	.59	.26	.53	.13	.70	.16	.57	.11
EJ	.49	.005	.45	.02	.32	.05	.57	.07
JM	.62	.21	.66	.15	.90	.32	.60	.11
Group	.69	.08	.62	.12	.71	.15	.59	.09
Tosyl arginine:								
LM	.79	.19	.71	.13	.61	.13	.63	.10
JC	.44	.14	.52	.13	.51	.11	.53	.25
RS	.68	.26	.55	.25	.70	.23	.54	.27
EJ	.67	.13	.39	.16	.43	.20	.48	.16
JM	.59	.21	.64	.19	—	—	.58	.19
Group	.66	.20	.60	.17	.57	.15	.55	.19

Therefore, this study demonstrates that the synthetic substrate methods as presently outlined are not suitable clinical methods for the detection of lysing or potential lysing blood. These results agree with the observations of Ratnoff(4) who found that the plasma proteolytic enzyme concentration, as measured by the casein method, did not differ between normal and fibrinolytic bloods.

Bastian *et al.*(5) have shown with the ester substrates that the esterase activity of trypsin and chymotrypsin are inhibited by rabbit plasma. The variation in demonstrable esterase activity in this study could be due to variations in the amount of inhibitor.

Summary. 1. An attempt was made to determine a normal level of enzyme activity in human plasma as measured by the lysine ethyl ester and tosyl arginine methyl ester assays. There was not only a variation in activity from one individual to another, but also variation in one individual from day to day and within one day. 2. When normal blood and blood with marked fibrinolytic activity were compared, no difference in enzyme activity was noted. Therefore, these methods are not suitable clinically for the

demonstration of fibrinolytic activity.

The authors wish to express their appreciation to Mr. E. D. Jenkins for the statistical analysis and to Dr. K. N. von Kaulla for his helpful suggestions.

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Evidence for Function of Aberrant Thyroid Tissue in Thymus of Rats.* (22899)

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In the course of a study on the long-term effects of massive doses of I^{131} in rats, aberrant thyroid tissue in the thymus was found in unexpectedly high incidence in the untreated controls(1,2). In 21 normal female rats of the Sprague-Dawley strain whose ages ranged from 260 to 427 days, one or more thyroid follicles were found in routine sections of thymus of 8 rats. Cords or sheets of epithelium resembling that of the thyroid were found in an additional 4 rats; in these latter there was no follicular arrangement or association with colloid. An attempt has been made to demonstrate whether the thyroid follicles in the thymus take up radioiodine and incorporate it in colloid.

Methods. Female rats of the Sprague-Dawley strain received 50 μc/g body weight of I^{131} when 55 days old and were autopsied 24 hours later. Histological sections were made of the thymus, and autoradiographs were prepared on Eastman Kodak 10 μ NTA stripping film.

Results. Fig. 1 illustrates the thymus of one of the rats at low magnification; thyroid follicles may be seen in the medulla. The tissue surrounding these follicles is characteristic of the thymic medulla. At higher magnification (Fig. 2) it is seen that these follicles possess a cuboidal epithelium and are filled with colloid. The colloid variably appears deeply eosinophilic and homogeneous or pale staining and vacuolated—the latter suggesting resorption. The follicles also vary in autoradiographic density (Fig. 3), but all show some radioiodine concentration. Thus, in structure and in iodine concentration, these follicles are like those found in the thyroid gland proper.

Discussion. The possibility that accessory thyroids and aberrant thyroid tissue may occur, especially in the mediastinum, has long been recognized. Adequate embryological explanations may be derived by study of the development of the branchial pouches which are the anlagen of both thyroid and thymus (3). It has been shown that administration of thiourea to Wistar strain rats resulted in a high incidence of thyroid adenomata in the thymus(4). The objective of the present communication is to point out a relatively high incidence of accessory thyroid tissue in an animal commonly employed in endocrinological experiments, and to show that this aberrant tissue may be functional rather than merely rudimentary. The findings have a bearing on experiments where total thyroid ablation is critical; surgical thyroidectomy may not reach all the functional thyroid tis-

* This work was performed under the auspices of the U. S. Atomic Energy Commission and the Committee on Research, University of California.

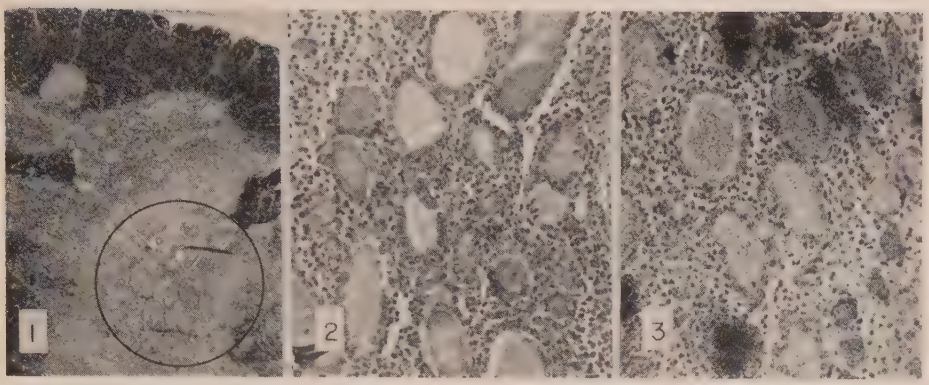

FIG. 1. Rat thymus; thyroid follicles in circled area. H and E × 20.
FIG. 2. Thyroid follicles from field in Fig. 1. H and E × 130.
FIG. 3. Autoradiograph of section of tissue neighboring that shown in Fig. 1 and 2. 10 μ NTA stripping film, 2 hr exposure, H and E × 130.

sue, and might have to be followed routinely by radioiodine. It would, in fact, appear possible that following surgical thyroidectomy the resultant increase in pituitary output of thyrotropic hormone could stimulate aberrant thyroid tissue. This possibility has been recognized, for example, in the studies by Entenman *et al.*(5) in which surgical thyroidectomy was followed by hypophysectomy.

Critical studies are under way to compare the thymi of surgically thyroidectomized rats with those of rats in which thyroid ablation was accomplished by radioiodine. Already differences in the oxygen consumption in the two groups of rats have been demonstrated by metabolimetry.

Summary. Aberrant thyroid follicles have been found in the thymi of female Sprague-Dawley rats. The accumulation of I^{131} by such follicles, shown autoradiographically, indicated their functional nature.

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Rape Oil and Cholesterol Metabolism in Different Species with Reference To Experimental Atherosclerosis.* (22900)

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Numerous studies on experimental atherosclerosis have provided ample evidence of differences in metabolism of cholesterol in different species of animals(1,2).¶ It is possible to produce marked hypercholesteremia and atherosclerotic lesions by feeding cholesterol to rabbits and chickens, and similar though less marked changes can be induced in guinea pigs. These effects can also be produced in dogs by concomitant administration of antithyroid compounds(3) and in monkeys by restriction of the intake of sulfur-containing amino-acids(4). Other species, notably the rat, appear to be particularly resistant to experimental atherosclerosis, although it is possible to increase the cholesterol content of blood and liver of the rat by giving bile acids and anti-thyroid compounds in addition to cholesterol(5,6).

While the rat is very resistant to production of atherosclerosis, and stores much less cholesterol than the rabbit when this compound is fed in the diet, studies in our laboratory have shown that rats fed diets containing rape oil deposit cholesterol in the adrenals and in the liver(7). Similar results were obtained by feeding erucic acid, one of the component fatty acids of rape oil(8,9). These diets, however, have little effect on blood cholesterol levels and no atheroma have been observed in the arteries after periods of 6 months or longer. It seemed of interest, therefore, to determine whether rape oil diets would cause more widespread deposition of cholesterol and formation of atheroma in species known to be susceptible to experimental atherosclerosis. This paper contrasts the results of feeding rape oil and of feeding cholesterol to animals of several different species.

TABLE I. Control Diets.

	Protein	Fat	Fibre
	%		
Masters fox chow	20(min)	3.5(min)	5 (max)
Purina rabbit chow	16.45	4.06	7.93
Grow mash	15(min)	3 (min)	7.5(max)

Methods. The rats used in these experiments belonged to the Sprague-Dawley strain, the mice were Swiss strain obtained from Rockland Farms, N. Y., and the chickens were White Leghorns from the Ontario Veterinary College, Guelph, Ontario. The guinea pigs, rabbits, cats and dogs were obtained locally. The dogs in the control group and all cats were females. Otherwise, male animals were used. The control diet for rats and mice consisted of Masters fox chow. Rabbits and guinea pigs were fed Purina rabbit chow checkers with supplement of cabbage twice a week, and the chickens received a Grow Mash prepared by the United Cooperatives of Ontario. The approximate composition of these diets is shown in Table I. The experimental diets were prepared by adding either 2% of powdered cholesterol or 25% of rape oil† to the control diets. The dogs were fed ¾ lb of Masters fox chow mixed with ¼ lb of rape oil once daily, together with a supplement of meat. All diets were fed for 28 days with the exception that the dogs were fed for 24 days. Cholesterol was determined by the Sperry-Webb procedure(10). Lipid extracts were prepared from single adrenals or 4 g samples of liver by grinding in acetone-alcohol(1:1) and extracting at boiling point with several portions of the same solvent. The extracts were made to measured volumes,

* This work was begun during tenure of a Fellowship from the Canadian Life Insurance Officers Assn. and was completed with the assistance of a grant from the Life Insurance Medical Research Fund. The experiments with chickens were made possible by the cooperation of Dr. J. S. Glover, Poultry Pathologist, Ontario Veterinary College, Guelph, Ontario. The author also wishes to acknowledge the valuable technical assistance of Mrs. Gordon Sanderson and Mr. E. A. Andersen.

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† Generous supplies of refined rape oil were donated by Yocum Faust, Ltd., London, Ontario.

filtered, and aliquots used for cholesterol determinations. In the experiment with mice, tissues from 2 animals were pooled for each determination. The coefficient of digestibility of rape oil was measured in rats, guinea pigs and rabbits by a method similar to that of Augur, Rollman and Deuel(11). Food consumption for animals on both control and rape oil diets was recorded for an 8-day period (4 days in guinea pig experiments). Combined feces for this period were collected and 10 g aliquots were ground and extracted overnight in Soxhlet with ether. The residue was acidified with 3 cc of concentrated hydrochloric acid and reextracted with ether. Total fat in feces was 105, 92 and 81 mg/g of stool in rats, guinea pigs and rabbits on control diets respectively, and these values were subtracted from amounts of fat found in feces on rape oil diets. Percentage of rape oil absorbed was calculated from the difference between amount ingested and corrected amount of fat excreted in feces.

Results. It is well-known that feeding diets containing cholesterol to rabbits results in hypercholesteremia and also leads to deposition of cholesterol in the liver, adrenal and other tissues(12,13). Such results were readily obtained in our experiments by feeding cholesterol as 2% of the diet for 28 days (Fig. 1). This diet produced less spectacular effects in guinea pigs but there were significant increases in cholesterol concentration ($P<0.02$ for plasma and $P<0.01$ for adrenal and liver). Cholesterol was not fed to chickens in the present experiments but Dauber and Katz(14) have shown that it increases blood cholesterol as in the rabbit. Their description of gross and microscopic appearance of tissues of treated chickens also suggests that lipids were being deposited in liver and perhaps in adrenals and other organs as well.

In species less susceptible to experimental atherosclerosis, feeding cholesterol has little effect. In our experiments, rats responded with a small increase in adrenal cholesterol and mice with some increase in liver cholesterol.

The results of feeding a diet containing

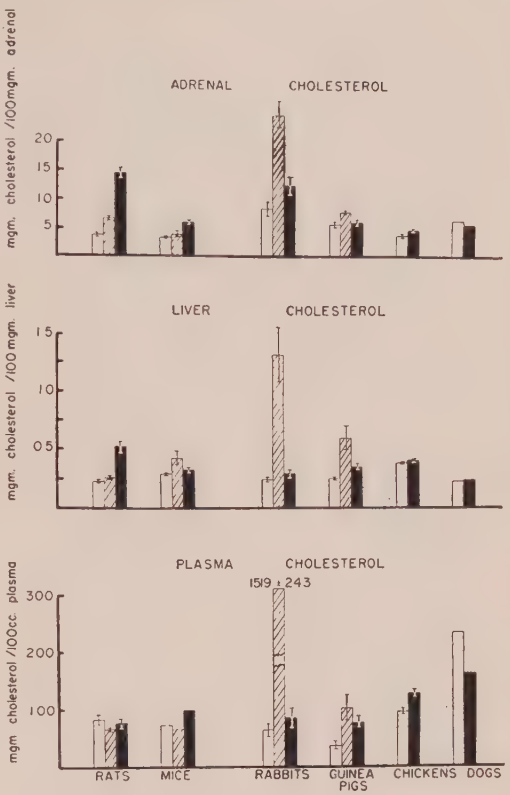

FIG. 1. Effects of different diets on plasma and tissue cholesterol concentrations. Open bars represent levels in animals on control diets; hatched bars represent levels on diets containing 2% cholesterol and shaded bars represent levels on diets containing 25% rape oil. Stand. error of mean is indicated in cases where it was determined.

25% of rape oil present a somewhat different picture. As had been shown previously, this regime caused a highly significant increase in adrenal and liver cholesterol in the rat ($P<0.001$), but no change in blood cholesterol. Adrenal cholesterol was also significantly increased in the mouse ($P<0.001$). (Insufficient determinations were made to test the significance of the increase in blood cholesterol). An attempt was also made to test the effect of rape oil on cats, another species which appears to be resistant to the production of experimental atherosclerosis. Results in this case were inconclusive because the cats refused to eat the rape oil diet and it was necessary to substitute a diet containing 10% of erucic acid, the active component of rape oil. Even this was poorly accepted and the

TABLE II. Effect of Diets on Body and Adrenal Weight.

	Control	Cholesterol	Rape oil	Control	Cholesterol	Rape oil	Control	Rape oil
	Rats			Mice			Dogs	
No. of animals	6	6	6	8	7	8	4	2
Initial wt (g)	84 ± 2.3	81 ± 1.0	84 ± 2.4	24 ± .5	24 ± .7	24 ± .8		
Final wt (g)	191 ± 9.7	186 ± 9.7	162 ± 3.9	30 ± .4	29 ± 1.3	26 ± 1.2	11,200	17,400
Adrenal wt (mg)	31.7 ± 1.2	30.8 ± 1.6	38.7 ± 1.2	4.6 ± .2	4.2 ± .1	4.4 ± .3	835	19,600
	Rabbits			Guinea pigs			Chickens	
No. of animals	5	5	5	6	6	6	13	14
Initial wt (g)	2230 ± 170	2330 ± 36	2580 ± 19	361 ± 12	373 ± 17	419 ± 14	1690 ± 55	1520 ± 45
Final wt (g)	2690 ± 152	2890 ± 60	2890 ± 108	482 ± 11	503 ± 14	407 ± 11	2550 ± 55	1900 ± 60
Adrenal wt (mg)	168 ± 17	292 ± 24	198 ± 8.8	241 ± 28	257 ± 16	223 ± 12	182 ± 8.5	135 ± 7.6

animals lost weight during the experiment. However, after 24 days on the diet, 2 of the 3 cats had more than 10% cholesterol in the adrenals whereas the adrenals of control animals contained only 4% cholesterol.

The rape oil diet appeared to have little or no effect on the adrenal cholesterol of rabbits, guinea pigs, chickens or dogs. There was, however, a tendency towards hypercholesteremia in guinea pigs ($P<0.01$) and chickens ($P<0.001$), and liver cholesterol was increased in guinea pigs ($P<0.001$). Rabbits, which are most susceptible to cholesterol diets, showed hardly any change in blood or tissue cholesterol as a result of feeding rape oil. Values for adrenal, liver and plasma cholesterol concentration are shown in Fig. 1, while adrenal and body weights are listed in Table II. It may be noted that animals on rape oil diet did not grow as well as those on control and cholesterol diets.

It seemed possible that failure of rape oil to affect adrenal cholesterol in some species might result from poor digestibility of the oil in those species, and to test this the coefficient of digestibility of rape oil was measured in rats, guinea pigs and rabbits. The values of 58, 61 and 58 which were obtained in these 3 species respectively, indicate that digestibility is probably not a factor in explaining species differences. These values are low compared to the figure of 82 obtained by Deuel, Cheng and Morehouse for rats(15). However, these workers used a synthetic diet containing 15% of rape oil, while in our experiments the diet consisted of fox chow to which 25% by weight of rape oil had been added. Rape oil from different sources has also been shown to vary greatly in its content of erucic acid(16) and this may account for some of the difference in digestibility.

Such short term experiments as those described above do not preclude the possibility that a rape oil diet may cause cholesterol plaques in a species such as the rabbit in a longer term experiment. However, in one experiment in which 3 rabbits were fed 25% rape oil for 72 days there was no evidence of formation of plaques in the aorta or coronary arteries. In another experiment, one group

of 5 rabbits was fed 2% cholesterol and a second group was fed 2% cholesterol together with 20% rape oil for 125 days. At autopsy these groups showed no difference in number or size of plaques in the aorta‡ and there were no significant differences in adrenal, liver and blood cholesterols of the two groups.

Thus it appears that rather than having an enhanced effect on cholesterol levels, rape oil fails to produce any marked changes in species susceptible to experimental atherosclerosis. There is, in fact, some indication of a negative correlation between susceptibility to experimental atherosclerosis and ability to respond to rape oil with an increase in adrenal cholesterol levels. It is conceivable that both of these characteristics may be related to species differences in the metabolism of phospholipids like those observed by Pilgeram and Greenberg(17).

Summary. Rape oil caused a marked increase in adrenal cholesterol when fed to rats or mice but had little or no effect in rabbits, guinea pigs, chickens and dogs. This contrasts with results obtained by feeding cholesterol since dietary cholesterol tends to be deposited more readily in the latter four species. The differences in response to rape oil do not appear to be due to species differences in digestibility of rape oil.

‡ The author is indebted to Dr. J. C. Paterson of the Department of Medical Research for examining the aortas and coronary arteries of these animals.

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Changes in Serum Proteins During Growth of Malignant Cells *in vitro*.* (22901)

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During periods of progressive growth of cells maintained as strains in continuous culture there is usually an initial adaptive growth followed by growth at near maximum rates and then a slowing down of growth but with the individual cells still active in fluid exchange, transport and storage. The products of these cellular activities may be expected to be reflected in the composition of the serum proteins when used as culture medium. The protein components appear to have importance in cellular responses not only because of their nutritional value but also in immune phenomena. Little is known how-

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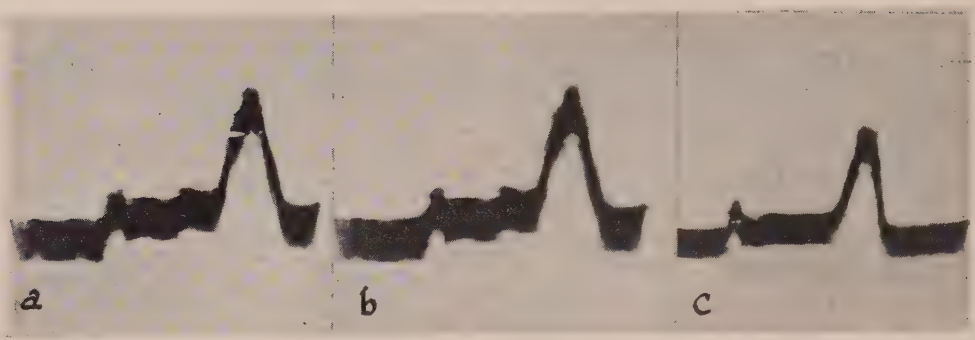

FIG. 1. *Schlieren diagrams* (electrophoresis): (a) *5050* fluid, (b) incubated *5050* fluid, (c) fluid from incubated cell cultures, TSAT-72.

ever about the cellular utilization of, or the contribution to, the serum proteins already present in tissue culture media during various periods of *in vitro* growth.

The role of the proteins in tissue culture has always been a difficult problem because of the complexity of these substances as they exist in biological fluids. We have carried out a series of preliminary studies in order to evaluate the changes in various serum proteins during continuous cell cultivation, especially in culture media derived wholly from such natural sources.

Materials and methods. The medium used in all the experiments was a mixture, designated *5050*, consisting of 50% human placental cord serum with 50% balanced salt solution (Gey). The continuously cultured cells tested were from the rat tumorous strains TSAT-72 and TSAT-333 which originated *in vitro* from normal rat mesenchyme, approximately 17 and 15 years ago, respectively. Also tested was the human fibrosarcoma strain A. Fi., explanted in 1938. Cultures of these strains were incubated for periods of 5 to 10 days without renewal of medium in standard 16 x 150 mm Pyrex roller tubes. The volumes of media used were carefully controlled. Three milliliters of culture fluid, *5050*, were used in these experiments with the cells grown directly on the tube wall. Colony sizes ranged from initial values of 5 mm with 4 colonies per tube to 8 mm at 5 days and 10 mm at 10 days. All experimental culture tubes were accompanied by 2 unincubated and 2 incubated *5050* fluid blanks without cells. These contained the same volumes of medium as their experimental partners and were run for the same periods of time. After 5 days, and as well after 10 days, the colonies and cells were observed to be healthy with many dividing cells present following this single fluid treatment.

Observations and results. At the end of the 5- and the 10-day periods, the culture fluids were centrifuged and the cell-free medium analyzed. Most of the electrophoretic studies were carried out on the fluids by the moving boundary technic, and as well by the paper zone microelectrorheophoresis technic. The Antweiler microelectrophoresis† and the Macheboeuf-Rebeyrotte‡ microelectrorheophoresis apparatuses were used. Electrophoresis with the Antweiler instrument was performed with a barbital buffer system at pH 8.6 with an ionic strength of 0.01. The Schlieren diagrams were photographed after 18 minutes migration under 70 volts potential and 1.8 milliamps. current (Fig. 1).

Significant depletion of the alpha and beta globulin fractions of the serum were noted in the fluids from all the cell cultures. The incubated *5050* fluid blanks did not show a variation in the protein pattern. Similar results were obtained with the technic of Macheboeuf(1). In this case the buffer was also Na-barbital-barbituric acid mixture at pH 8.6 with an ionic strength of 0.05. The duration of migration was 4 hours under 255 volts and 0.5 milliamp. current for one square centimeter of paper. The protein pat-

† From I. Sorvall, Inc., Norwalk, Conn.
‡ From Laboratoires Leres, Paris, France.

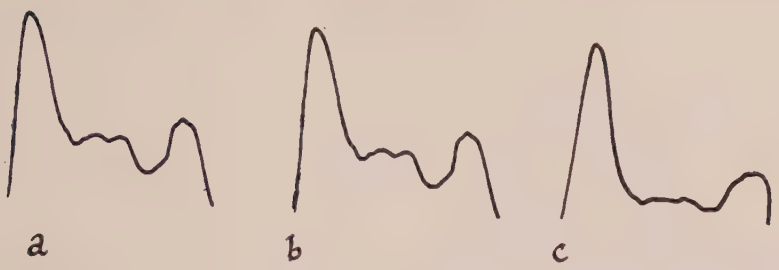

FIG. 2. *Paper electrophoresis recordings:* Same as in Fig. 1.

terns were photographed from the paper strips with an automatic recording photometer‡ (Fig. 2). Here again the decreases in the alpha and beta globulins were noticed in all the cell culture fluids. The albumin fraction seems to remain unchanged. As can be seen from these results, "the alpha-beta reduction phenomenon" appears to be a reproducible one and there is a general agreement in the patterns obtained by the two different procedures.

To support the electrophoretic studies on a more quantitative basis, the Folin-Ciocalteau-biuret procedure described by Lowry *et al.* (2) and the classical microanalytical procedure of Kjeldahl were used for the determination of the proteins and the total nitrogen consecutively. The diagrams (Fig. 3) show the decreases in proteins and also total nitrogen content of the media obtained under these experimental conditions. These microanalytical values support the electrophoretic observations that a significant reduction of the alpha and beta globulins accompanies *in vitro* cell growth during the 5- and 10-day periods.

In a limited number of preliminary tests with pseudo and euglobulins isolated from human placental cord serum and used as the only source of nitrogen in the culture medium, there was also evidence, in both cases, of a reduction of the alpha and beta globulins. Other direct culture tests with alpha globulins isolated by electroconvection showed a significant reduction of these proteins in the cell cultures of the TSAT-72 rat tumorous strain. Fig. 4 illustrates the results of this particular experiment.

Discussion. One might think that these proteins had undergone proteolysis under the influence of enzymes liberated by the growing cells in the culture fluid. However, the total nitrogen values do not support this possibility unless the peptides or the amino acids released by a postulated proteolysis were incorporated by the cells immediately. On the other hand, without cells present the incubated blanks of *5050* fluid showed no evidence of proteolysis. Microchromatographic studies for amino acids with such fluids incubated for 2, for 3, and for 4 days, even in the presence of growing cells, do not reveal any significant increase in the free amino acids already present, nor any appearance of other amino acids. There still remains the question of whether the protein molecules can be incorporated by the cells as such or whether they are first broken down into smaller frag-

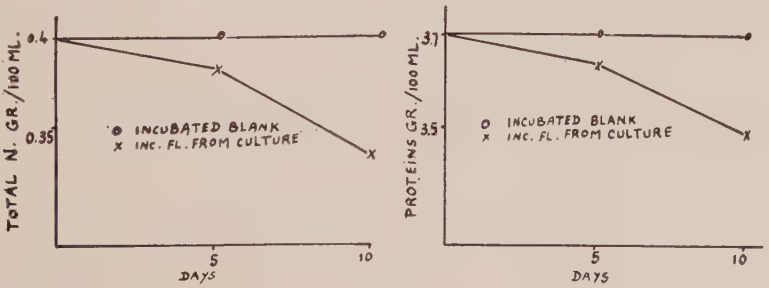

FIG. 3. Decrease in protein and nitrogen of TSAT-72 culture fluid.

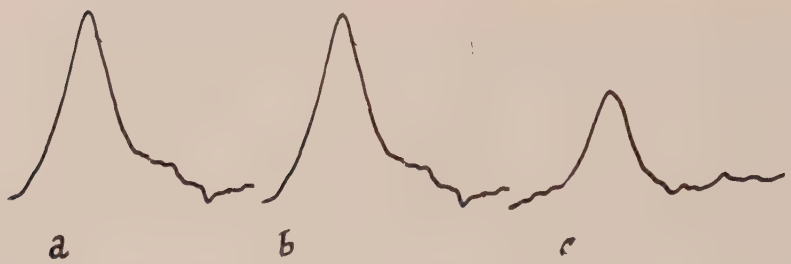

FIG. 4. *Paper electrophoresis recordings:* (a) Alpha globulin medium—no cells, (b) incubated alpha globulin medium—no cells, (c) decrease in alpha globulin when used as culture medium with cells.

ments extracellularly. Attractive experimental data exist concerning the incorporation of whole proteins as shown by Coons(3), as well by Gitlin and Whipple(4) and also by Winnick(5,6) in a series of interesting studies. Using human placental cord serum Jaquez and Barry(7) observed the growth promoting activity of the human placental cord serum which we have used in our laboratories for over 25 years, and reported that this activity is associated with the nondialyzable proteic fraction; the globulins in general had all the activity of the serum. Madden and Whipple's experiments(8) on the participation of serum proteins in the metabolism of cells show another example of the utilization of these substances by living cells *in vivo*. Concerning the method of incorporation of these macro-molecules, one has little difficulty in establishing protein incorporation by the pinocytosis phenomenon of Lewis(9, 10). Other workers claim to have demonstrated extracellular breakdown of proteins before or during incorporation(11,12).

According to our data, apparently entire molecules of protein may be taken up by cells in continuous cultures, yet one must assume that some structural modifications occur during the incorporation. These studies are by no means complete, yet the results appear to be consistent and therefore have considerable significance if we are to understand the method of incorporation of these components into cells and the cellular donation to the external milieu. Some of the future work will be directed toward the use of labelled components in these incorporation studies carried out with living cell systems and will be coupled with quantitative procedures already in use.

Summary. The changes that occur in the serum protein fractions of the tissue culture medium during growth of 2 rat and one human tumorous cell strains maintained in continuous tissue culture were studied. Electrophoretic studies on the culture fluids by the moving boundary, and as well the paper electrophoresis technics, revealed a significant decrease of the levels of alpha and beta globulins of the serum nutrient during specific periods of growth. These findings are supported by microanalytical data.

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Electrocardiographic Patterns of Normal and Thiamine-Deficient Baby Pigs.* (22902)

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Electrocardiographic (ECG) studies of normal and thiamine-deficient individuals in man and several animal species have been made and are well reviewed by Lepeschkin(1). Studies with a limited number of animals(2-4) have dealt with ECG changes in thiamine-deficient pigs beyond weaning age. The thiamine requirement of the baby pig has been reported by the present workers(5). Electrocardiograms were obtained but were not reported. We have noted no communications in which the ECG pattern of either normal or thiamine-deficient baby pigs has been submitted. The purpose of the present paper is to present such patterns and to submit a summary of duration data for various electrocardial intervals.

Methods. Electrocardiograms were made weekly on 35 baby pigs which were taken from the sow when 3 or 4 days of age and placed in individual cages where they received a synthetic milk diet. These pigs were used during the second and third replications of the thiamine requirement study(5). Diet and controlled environment have been described previously(5,6). Seven pigs received no thiamine in the diet and developed acute thiamine deficiency. Fourteen pigs received 0.5 to 1.0 mg thiamine/kg of dietary solids. These animals developed milder deficiency symptoms. The remaining 14 baby pigs received milk containing 1.5 to 2.0 mg thiamine/kg of solids in the diet and these pigs were quite normal in all respects. To obtain valid ECG data the pigs were suspended in a canvas carrier device. With some training the pigs learned to lie still for a considerable period of time. Recordings were not made until the pigs had remained still for a period of 3 minutes or more. The electrocardiograph employed was a 4 channel, model 67 Sanborn. Five leads were connected to electrodes situated on the 4 legs and the chest. Recordings were made from the 3 standard dipolar leads, the unipolar AVR, AVL, and AVF leads and the unipolar chest lead. A comparison of

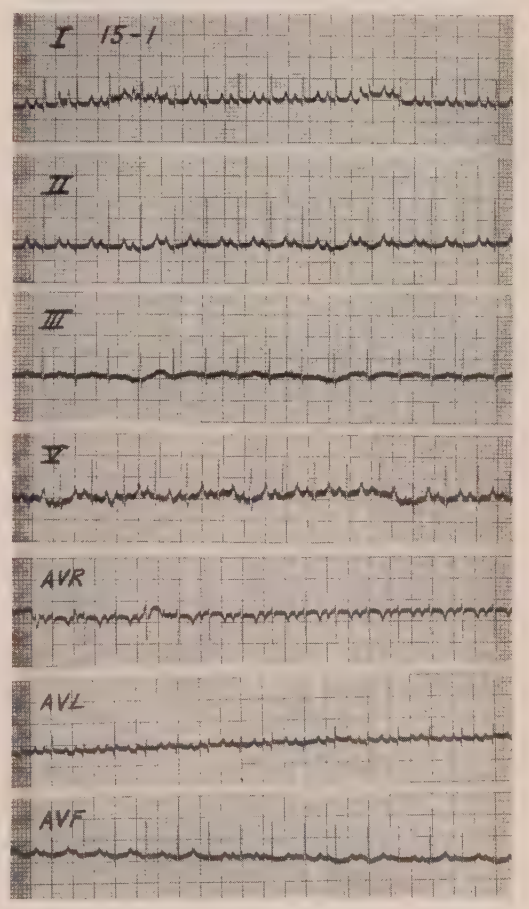

FIG. 1. Seven lead recordings made on all electrocardiograms. This ECG is from one control pig during fifth week. Heart cycle length is uniform, heart rate is 190 bpm, PR time is 0.08 sec. and QRST duration is 0.20 sec.

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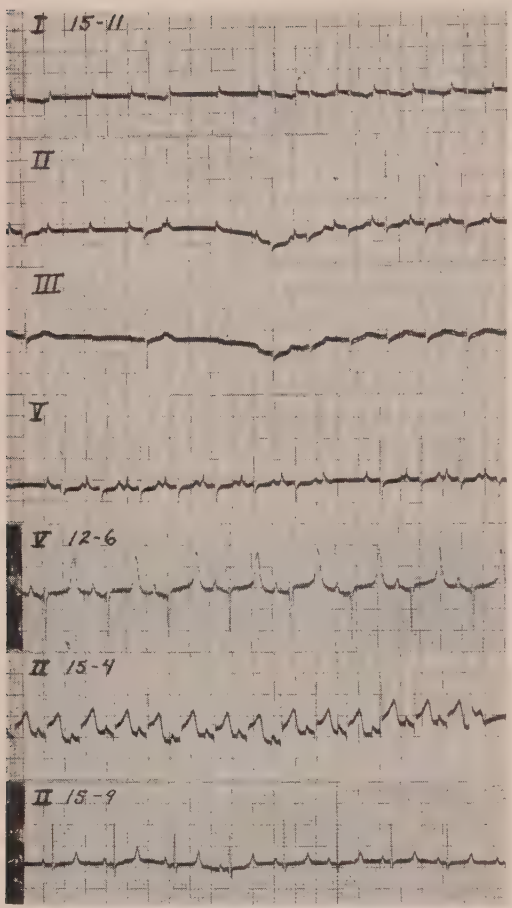

FIG. 2. These tracings from thiamine deficient pigs reveal varying degrees of bradycardia in all pigs. A marked sinus arrhythmia is exhibited by pigs 15-11 and 15-4. First degree AV block is apparent in pig 12-6 and second degree AV block is exhibited by pig 15-11. Duration of QRST interval is excessive in all pigs except 15-4 and PR time is excessive in pigs 12-6 and 15-11. ST segment is greatly elevated in pig 15-4 and T wave potential is abnormally high in pigs 12-6, 15-9, and 15-4.

duration of ECG intervals between normal and thiamine-deficient animals was made. These data were taken from tracings which were made during the fifth week of age or from the ECG last obtained before death in case of deficient animals which died. These data were statistically analyzed using the method for single classification variance(7).

Results. Electrocardiograms from normal animals usually showed positive P, R and T waves in the standard leads although T was sometimes inverted in all of these leads and very often inverted or small in lead I and often barely perceptible in lead III. P was often barely discernible and sometimes inverted in lead III. The S wave was usually not present in lead I. There were no particular changes in this pattern from birth to 5 weeks of age. There appeared to be no consistent differences between normal and thiamine-deficient animals in the direction of any waves in the standard leads (Fig. 1 and 2) although in several of the acutely deficient pigs the T wave voltage greatly exceeded that found in normal pigs. T wave voltage was normally less than 0.2 millivolt in all leads.

The P, R, and T waves were usually all inverted in the AVR unipolar lead. In the AVL lead T was usually inverted and both P and T were often small or not perceptible. T was occasionally inverted in the AVF lead. T was usually of greater magnitude from the unipolar chest lead than on any other lead. Again, no consistent changes in unipolar lead tracings, either in wave magnitude or direction were effected by advancing age or thiamine deficiency.

Duration of heart cycle in control pigs was usually uniform although a slight degree of sinus arrhythmia appeared not to be abnormal. A marked sinus arrhythmia was observed in most of the deficient pigs. Bradycardia was exhibited by all pigs which developed an acute thiamine deficiency.

A summary of duration of ECG intervals is presented in Table I. The PR interval was significantly lengthened in acutely deficient pigs. In one of these pigs (Fig. 2) this interval was 0.14 second which probably indicates first degree auriculoventricular (AV) block. The QRS interval was not lengthened in thiamine-deficient pigs and was consistently 0.04 second in duration. The period of electrical systole (QT interval) was significantly lengthened in both chronically and acutely deficient pigs. In one of the acutely deficient pigs (Fig. 2) the ventricular phase of the cycle was occasionally absent probably because of second degree AV block. A comparison of Bazett's constant(8) reveals that the systolic portion of the cycle constituted a relatively greater amount of the cycle duration in the deficient pigs.

TABLE I. Duration* of Electrocardial Intervals in Normal and Thiamine-Deficient Baby Pigs.†

	Normal	Acute thiamine deficiency	Chronic thiamine deficiency
No. of pigs	14	7	14
PR interval	.08 ± .00‡	.10 ± .01¶	.08 ± .00
QRS	.04 ± .00	.04 ± .00	.04 ± .00
ST	.10 ± .01	.16 ± .02‖	.12 ± .01
QT	.20 ± .01	.27 ± .01¶	.23 ± .01‖
RR	.33 ± .01	.48 ± .04¶	.35 ± .01
Bazett's constant§	.35 ± .01	.39 ± .02‖	.39 ± .01‖

* Interval time durations expressed in sec.

† Data for normal pigs was taken from ECG made at 5 wk of age. Data for deficient pigs was obtained at similar age or from ECG just preceding death in the case of those which died.

‡ Stand. error of mean.

§ $\frac{QT}{\sqrt{RR}}$.

‖ Significantly greater than for normal pigs ($P = 0.05$), and ¶ ($P = 0.01$).

Duration of heart cycle and each electrical interval was increased somewhat in normal pigs as their age advanced. Heart rates dropped from average of 266 bpm for the first week to average of 189 bpm by the second week and then reduced only gradually thereafter to average of 180 bpm at 5 weeks of age. The PR interval increased from 0.06 second shortly after birth to 0.08 second at 5 weeks of age. The ST interval increased from 0.06 second to 0.10 second and the QT was increased from 0.14 second to 0.20 second over the same period. The QRS interval and the interval of the T wave remained quite constant in duration over this age period and were 0.04 second and 0.06 second respectively.

Three pigs in the group receiving no dietary thiamine died suddenly during third and fourth week after having exhibited severe degree of anorexia and depressed growth rate. The remaining 4 pigs from this group also showed these acute symptoms and 2 of these were sacrificed *in extremis* during the fifth week. The other 2 pigs from this group recovered following intraperitoneal injections of thiamine hydrochloride. Although the effect on dietary intake and growth depression was not nearly so great in pigs receiving 0.5 mg or 1.0 mg of thiamine/kg of dietary solids, 3 of these pigs died during the fifth week and analysis of heart weight data from animals which were sacrificed at this time indicates that a greater degree of cardiac hypertrophy had occurred in this group than in pigs from the group receiving no thiamine. The ECG pattern of the lead II tracing for pig 15-4 of this group is shown in Fig. 2.

Summary. Electrocardiograms were made weekly from birth through 5 weeks of age on 35 baby pigs receiving various subminimal and adequate levels of thiamine in the diet. Typical ECG patterns of normal and thiamine-deficient baby pigs are presented and described. Statistically significant increases in PR time, QT time and cycle length occurred in deficient animals. Thiamine-deficient pigs consistently exhibited sinus arrhythmia. First and second degree auriculoventricular block were manifested. Evidence of cardiac hypertrophy occurred in deficient animals.

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Intermediary Metabolism of Antibiotic Resistant and Antibiotic Sensitive Staphylococci. I. Pyruvate, Glucose and Acetate.* (22903)

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In an earlier study, Fusillo(1) noted that *Micrococcus pyogenes var. aureus* (staphylococci) which were naturally resistant to penicillin, streptomycin, and the tetracyclines appeared to exhibit a decreased exogenous requirement for thiamin and/or nicotinamide. One might conclude from this study either that the organism is synthesizing its own vitamin requirement, compensating for this vitamin need, or that the enzyme complexes associated with thiamin and/or nicotinamide are no longer active. Padron and coworkers(2) in metabolic studies with *in vitro* selected chloramphenicol resistant organism supported the view that the latter enzymes were inoperable. However, in the former study(1) when thiamin and/or nicotinamide were added to the basal medium, stimulation of growth of resistant organism occurred. It was considered(1), that the vitamin associated enzyme systems were still operative. This report elaborates some observable differences in the metabolism of sensitive and naturally selected resistant organisms and presents evidence which reveals the continued existence of potentially active thiamin and/or nicotinamide associated enzymes in resistant cells.

Methods and materials. The sensitive strain used was *M. pyogenes var. aureus* (ATCC 9996) and the multiple resistant organism used was isolated from a burn wound, (Stickle). Stock cultures were carried on trypticase soy agar slants. Organisms for whole cell preparations were grown 16 to 18 hours at 37°C in trypticase soy broth. Organisms for frozen and thawed lysed cell preparations were grown on trypticase soy agar. Cells were harvested and washed 3 times in M/15 phosphate buffer, pH 6.8. Cell suspensions were adjusted to an optical density to contain 14 mg dry weight of cells per ml of suspension. Two ml of this suspension were used in each Warburg vessel. Oxygen uptake was determined with 10% KOH in the center wall, and air as the gas phase by conventional Warburg technics. Final volume in each flask was 2.6 ml. Whole cell preparations were refrigerated 18 hours at 4°C to reduce extremely high endogenous respiration.

TABLE I. Oxidation of Glucose, Pyruvate and Acetate by Whole Cell and Lysed Cell Preparations of Staphylococci.

	μl O_2 consumed/hr			
	Sensitive cells		Resistant cells	
	Whole	Lysed	Whole	Lysed
Glucose*	191	—	193	—
Pyruvate*	55	52	0	42
Acetate*	0	0	0	0

* Substrates 0.02 mM, dry wt cells 28 mg. All values corrected for endogenous respiration.

Results. Glucose was oxidized by both sensitive and resistant cells to the same extent. (Table I).

Whole cell preparations of sensitive cells oxidized pyruvate, whereas resistant cells did not. The resistant cells, when lysed, were found to oxidize pyruvate.

Cocarboxylase did not activate oxidation of pyruvate by whole resistant cells (Table II). Oxidation of pyruvate in lysed resistant

TABLE II. Effect of Co-factors on Pyruvate Oxidation by Whole and Lysed Resistant Cells.

	μl O_2 consumed/hr	
	Whole	Lysed
Pyruvate	0	42
" + cocarboxylase	0	43
" + Mg^{++}	0	70
" + " + cocarboxylase	0	101

Pyruvate and Mg^{++} 0.02 mM, cocarboxylase 2 μg. Dry wt of cells 28 mg. All values corrected for endogenous respiration.

* This investigation was supported by Office of the Surgeon General, Department of Army, contract DA 49-007-MD 724.

cells was not greater than the controls when cocarboxylase was added to the system. Magnesium ions stimulated oxidation of pyruvate in lysed resistant cell preparations but were without effect on whole resistant cells. In the presence of Mg^{++} and cocarboxylase, oxidation of pyruvate was further stimulated in the lysed resistant system. Acetate, malate, and acetaldehyde were not oxidized by any of the systems used.

Discussion. The inability of whole cell preparations of naturally selected multiple resistant staphylococci to oxidize pyruvate is similar to the findings of Ramsey and Padron(3) who used *in vitro* selected chloramphenicol resistant staphylococci. Ability of lysed preparations of these cells to oxidize pyruvate confirms the observations of Fusillo (1) that thiamin and/or nicotinamide enzyme complexes were still present.

The experiments reveal that Mg^{++}, which in sensitive cells would activate the carboxylase system, does not appear to be available for that purpose in resistant cells. Magnesium did not enhance pyruvate oxidation by whole cell preparations, but stimulation was evidenced in lysed resistant cell preparations. Weinberg(7) observed a reversal of inhibitory effect of oxytetracycline on growth of sensitive bacteria by Mg^{++} and other divalent cations. Also, he postulated that oxytetracycline might act as a chelating agent for the divalent ions. It follows from our experiments that in resistant cells, either Mg^{++} is not assimilated or that Mg^{++} is bound as it is assimilated. Whether Mg^{++} ions are bound as ribonucleate, as observed by Gale in penicillin resistance studies and whether this binding is reversible warrants further study. Chelation of Mg^{++} would explain inability of the tetracycline resistant organism to produce an anti-tetracycline enzyme comparable to penicillinase.

Conflicting views on acetate oxidation by staphylococci are apparent in the literature. Stedman and Kravitz(4) and Padron *et al.* (2) claim oxidation of acetate occurs while Wolin *et al.*(5) and the above studies noted the lack of oxidation.

Concerning the staphylococci, these and former findings further develop the concept that the steps involved in the dissimilation of pyruvate appear to be a major point of antibiotic attack, and that perhaps two(6) or more pathways are available for pyruvate dismutation. Multiple potential metabolic pathways that exist before the antibiotic block occurs in the normally favored pathway would explain the *a priori* existence of single and multiple antibiotic resistant variants of the staphylococci(8).

Summary. Evidence is presented that while whole cell preparations of antibiotic resistant staphylococci did not oxidize pyruvate, lysed cell preparations did. Magnesium ions and Mg^{++} plus cocarboxylase, but not cocarboxylase alone, stimulated pyruvate oxidation by lysed cells, but had no effect on the activity of whole cells. Activity of isolated cell free extracts, cell surface components, and chelating ability of tetracycline are being studied.

The authors acknowledge the very capable technical assistance of Mr. Martin Morris and Miss Carroll Possehl.

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A Factor in Human Gamma Globulin Preparations Active Against *Pseudomonas aeruginosa* Infections. (22904)

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Pseudomonas aeruginosa exhibits low virulence for mice, producing death only when injected intraperitoneally in large numbers (10^8 to 10^9 organisms). An endotoxin probably plays a part in these deaths, which occur chiefly within 12 to 24 hours.

The susceptibility of mice to Pseudomonas can be enhanced by pretreatment with cortisone, so that smaller inocula can be used(1). Under these conditions a more delayed mortality is produced, affording a more suitable technique for *in vivo* studies of antibacterial agents. By this procedure it has been possible to demonstrate a potent therapeutic effect from normal human γ globulin preparations, while human serum albumin is devoid of this principle.

Methods. Female albino mice of the NIH general purpose strain, weighing 17 to 22 g were used. Two strains of *Ps. aeruginosa,* isolated from patients with burns(2), were employed. They have been classified by Dr. Elizabeth Verder of the National Microbiological Institute as serological types 10: 1, 2. Lyophilized milk suspensions of the bacteria were inoculated into meat extract broth and

TABLE I. Results in Mice Pretreated with Cortisone. γ-globulin, albumin and plasma were all from human sources. One inj. only, within 3 hr after infection, unless otherwise specified. Organisms intraperitoneally.

Therapy	No. of organisms	No. of mice	% mortality — Days after infection						
			1	2	3	4	5	6	7
A. Controls, untreated	8×10^5	110	13	50	63	71	72	76	84
B. 16% γ-globulin									
.2 ml i.v. × 2	8×10^5	25	4	4	4	8	16	20	24
.2 ml i.v.	"	29	3	10	10	24	24	31	31
.1	"	45	2	9	13	13	16	18	20
.05	"	15	0	7	20	27	27	33	40
.02	"	29	0	7	10	14	14	14	14
.005	"	14	0	21	28	50	50	50	57
.002	"	23	0	22	30	35	39	48	57
.0005	"	15	0	13	33	33	47	53	67
C. Controls, untreated	8×10^6	20	30	70	75	75	80	80	80
γ-globulin, .1 ml i.v.	"	14	0	0	0	7	14	36	36
" .007	"	9	0	11	22	22	33	33	45
" .002	"	10	0	30	40	40	60	60	60
D. Plasma, .3 ml i.v. or i.m.	8×10^5	26	12	19	31	35	39	46	50
Plasma, heated, 30 min. i.v. or i.m.									
56° — .3 ml	8×10^5	26	4	15	23	23	23	27	31
65° — .3	"	25	8	28	32	36	44	48	52
85° — .3	"	12	25	58	67	75	75	83	83
E. 16% serum albumin i.v.									
.2 ml i.v. × 2	8×10^5	15	13	53	60	60	73	73	73
.2 ml i.v.	"	10	10	70	70	80	90	100	100
F. 16% γ-globulin									
.2-.4 ml i.v.	0	10	0	10	10	10	10	20	20
Plasma, .3 ml i.v.	0	9	0	0	0	0	0	0	0
Cortisone only	0	28	0	4	7	7	7	7	7
Organisms only	8×10^5	155	0	0	0	0	0	0	1

i.p. = intraperitoneally; i.m. = intramuscularly; i.v. = intravenously.

incubated for 5 hours at 37°. After centrifugation the organisms were suspended in 0.9% NaCl to an optical density of 0.40 to 0.45 (at 650 mμ) representing an approximate concentration of 1.7×10^9 organisms per ml. This suspension was further diluted with saline 10^2 to 10^3 fold. Mice received intraperitoneally 0.5 ml of the diluted saline suspension (approximate infective dose, 8×10^6 to 8×10^5 organisms). *Cortisone pretreatment* of mice consisted of 5 intramuscular injections (0.1 ml) of 1.25 mg each of cortisone acetate at daily intervals beginning 4 days prior to infection, the last dose being given on the morning of inoculation. Under these conditions 80 to 100% of the animals died within 10 days, the majority of deaths occurring between the second and fifth days. Control animals receiving cortisone alone or bacteria alone have only occasionally died(1).

*Human γ globulin** was injected intravenously, intramuscularly or intraperitoneally within 3 hours after bacterial inoculation, in a volume of 0.1 to 0.2 ml; only one dose was given, unless otherwise specified. Dilutions of the original γ globulin, containing 16% protein, were made in 0.9% NaCl. Normal human serum albumin, 25%, was similarly diluted.

Results. Cortisone-treated mice. Very small amounts of γ globulin afforded a high degree of protection. Thus, a single intravenous injection representing 0.002 ml of the original 16% solution, or 320 μg of protein, protected approximately half the animals, while 0.02 to 0.2 ml prevented death in 70 to 86% of them (Table I, B; Fig. 1A).

While a high degree of protection was afforded by 0.02 ml, complete protection was not always attained by increasing the dosage of γ globulin 10 to 20 fold, in single or multiple injections. Some deaths from large doses may be due to extraneous toxicity of the cortisone plus globulin (Table I, F), since blood cultures were not taken on all fatalities. It

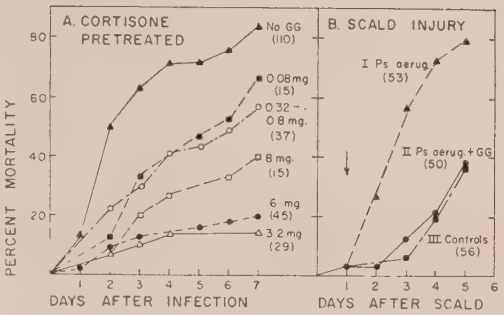

FIG. 1. Protective action of γ-globulin (GG) on Pseudomonas infections. A. Mice pretreated with 5 intramusc. injections of 1.25 mg of cortisone; organisms intraper. on fifth day, followed within 3 hr by varying amounts of GG intrav. as indicated. B. Susceptibility of thermally injured mice to Pseudomonas infections, and response to GG. Infection intraper. in Groups I and II (arrow), the day after injury; 16 mg GG intramusc. in Group II one hr after inoculation (8×10^6 organisms). Figures in parentheses = No. of mice.

should also be pointed out that heterologous γ globulin rapidly disappears from the circulation of the rabbit after a period of 4 or 5 days, due to antibodies formed by the host against the foreign protein. Therefore, with the use of a heterologous protein, the protective substances may be antagonized after the first few days. The *protective action* is manifest against many lethal doses of organisms, since increasing the inoculum 10 fold (8×10^6 organisms) did not greatly alter the results (Table I, C).

Human citrated plasma pooled from 3 donors, in dosage of 0.3 ml, intravenously or intramuscularly, gave a degree of protection in cortisone-treated mice consistent with its γ globulin content; experiments are in progress to determine the relative titre of plasma, and whether its activity is confined to the γ globulin fraction. A sample of chromatographically purified γ globulin from fresh human serum(3)† gave a degree of protection similar to that of commercially prepared material. Preliminary studies with normal mouse plasma (NIH strain) revealed little or no protective action in doses of 0.3 ml intravenously. To test the heat stability of the factor, samples of human plasma were heated for 30 minutes at 56°, 65° and 85°. The

* γ globulin preparation was kindly supplied by American Red Cross. This preparation is marketed as poliomyelitis immune serum globulin (human). Electrophoretically it is 99.1% γ globulins and 0.9% β globulins.

† Kindly supplied by Dr. Herbert A. Sober of National Cancer Institute.

TABLE II. Effect of γ-Globulin on Pseudomonas Infections in Normal Mice (No Cortisone Administered). γ-globulin inj. i.p. 3-6 hr before bacterial inoculation, to allow time for absorption.

Therapy	No. of organisms	No. of mice	% mortality — Days after infection						
			1	2	3	4	5	6	7
Controls, untreated	8×10^7	20	80	83	83	83	83	83	83
	1.6×10^7	20	5	5	10	10	10	10	10
	8×10^6	40	0	0	0	0	0	0	0
γ-globulin, 16% i.p. 3-6 hr before inoculation									
.1 ml	8×10^7	20	33	38	38	38	38	38	40
.1 ml	1.6×10^7	20	0	0	0	0	0	0	0

sample heated at 56° was centrifuged at 3000 r.p.m. to remove precipitated proteins, and the supernatant was injected intravenously or intramuscularly. Samples heated at 65° became gelatinous, while those at 85° developed a heavy precipitate; these were injected (without centrifugation) intraperitoneally or intramuscularly. Under these conditions activity was retained in the samples heated at 56° and 65°, and was lost at 85° (Table I, D). Experiments with normal human serum albumin injected intravenously in doses comparable to the highest amount of γ globulin employed, showed it to be devoid of protective action (Table I, E).

Normal mice. A few experiments were carried out on the antagonism in mice without cortisone pretreatment. An inoculum of 8×10^7 organisms intraperitoneally was required to kill normal mice. 0.1 ml of γ globulin injected intraperitoneally 3-6 hours before bacterial inoculation reduced the mortality from 83% in the untreated group to 40% (Table II).

Experiments with thermal trauma. Systemic infection with *Ps. aeruginosa* and other organisms normally of low virulence is a frequent sequel to extensive burns and other trauma. In mice subjected to a standardized trauma, in which acute death from shock was prevented by therapy with isotonic saline solutions, a high percentage of animals succumb within 2 to 3 weeks(4). In the present experiments a somewhat less severe degree of trauma was employed (unshaved etherized mice, dipped to the axilla for 6 seconds in water at 70°) in an attempt to retard the incidence of delayed deaths. Two ml of 0.9% NaCl was administered immediately after the trauma to prevent death from shock. The following day the mice were divided into 3 groups. Group I received intraperitoneally 0.5 ml of *Ps. aeruginosa* (8×10^5 organisms); Group II received organisms intraperitoneally and 0.1 ml of γ globulin intramuscularly within an hour following infection, and Group III served as a control group (injury only). In this way it was possible to test susceptibility of thermally injured mice to *Pseudomonas* infections, as well as the effect of γ globulin therapy upon infection.

Most of the traumatized mice injected with an inoculum of *Pseudomonas,* non-fatal to normal animals, succumbed within the first 4 days, while the mortality curve of those receiving γ globulin was much lower, and similar to that of the control group (Fig. 1B). Many of the animals in Groups II and III died at a later interval, and the cause(s) of death require elucidation. It remains to be determined whether more prolonged treatment with a homologous antibody will influence these later deaths.

The results with *Pseudomonas* infections in burns are similar to those obtained with the use of cortisone, and suggest that in extensive trauma the susceptibility to infections of this type may be related to excessive secretion of steroid hormones by the animals, as a consequence of the trauma. The above experiments also suggest that γ globulin therapy may be of benefit in *Pseudomonas* infections subsequent to trauma.

Discussion. Further work is in progress to supplement the present incomplete report. The nature of the protective mechanism, and

its application to other bacterial infections, must await future experimentation. Landy and Pillemer have shown protection against *Pseudomonas* and other Gram-negative bacterial infections by prior injection into mice of lipopolysaccharides that increase the properdin titre of blood serum(5). However, properdin is reported to be in Fraction III (Cohn) of the plasma proteins(5), while γ globulin is obtained from Fraction II. Likewise, heating serum for 30 minutes at 56° destroys properdin titre(5), while in our experiments with plasma no loss of activity occurred.

Summary. A factor in the γ globulin fraction of human plasma has been shown to prevent death in mice from *Ps. aeruginosa* infections, when injected subsequent to the inoculation. Protection was demonstrated in normal mice, and in mice rendered more susceptible to this infection by pretreatment with cortisone, or by subjecting them to thermal trauma. Human serum albumin was without activity.

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Tissue Heparin and Mast Cells in Rats and Rabbits.* (22905)

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It is generally assumed that heparin is synthesized and stored in the mast cells, and that variations of mast cell numbers and of heparin contents are parallel, when different tissues are compared(1,2,3). Recently species differences in susceptibility to atherosclerosis were correlated with differences in mast cell numbers; the greater resistance to this disease of certain species such as, *e.g.*, the rat, was attributed to heparin being available in relatively greater quantities, since this anticoagulant was shown to accelerate removal of certain plasma lipids, via the clearing factor(4). To test this hypothesis, tissue heparin concentrations and mast cell numbers were compared in species resistant and susceptible to atheromatosis, *i.e.*, the rat and rabbit.

Material and methods. Twenty adult male rats of the University of S. California strain, fed Purina Chow, and 12 adult male albino rabbits obtained from a commercial breeder, were used without further treatment. The animals were anesthetized with nembutal, and liver, intestine, kidneys, lungs, spleen and thymus were excised and weighed. From each of these organs, a small slice was removed for histological examination; samples of 1-5 g were then taken for heparin extraction, weighed and stored in the frozen state. Heparin was extracted, defatted, deproteinized by tryptic digestion and partially purified according to a method adapted to relatively small samples, and relatively low heparin concentrations, as reported recently (5). The heparin content of the extracts obtained was measured in line with a semimicro

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modification of the procedure described in the U.S. Pharmacopeia XIV. Duplicate samples were run on many of the larger organs, in particular, those derived from rabbits. In the case of small organs such as spleen and thymus, however, samples of several animals were usually pooled.

The combination of extraction and assay procedures described here is considered to be specific for heparin, since the determination is based on anticoagulant activity, and as no other known naturally occurring anticoagulant can survive the treatment involved, as far as we know. To further establish the identity of the heparin obtained, a number of characteristic tests were carried out, including protamine-reversal of anticoagulant activity, paper electrophoresis, and metachromatic activity of the product obtained in the latter procedure. According to these criteria, the heparin of the tissue samples was identical with a reference preparation of heparin sodium USP, as reported recently(5). For microscopic examination, tissue samples were fixed in a mixture of 1 part formalin and 2 parts 80% (v/v) alcohol, embedded in paraffin and cut at 5 μ. Fixation of rabbit material with basic lead acetate proved unnecessary. Various basic dyes were tried for staining. For routine use 1% aqueous toluidine blue was chosen. Rat sections were stained for 10-15 minutes, those of rabbits for 15 minutes; the slides were then quickly dehydrated with isopropylalcohol. Eight to 10 days later, metachromasia was well developed. Most of the rat mast cells turned purplish-black; a few remained pink. Very tightly packed granula, *e.g.*, in the elongated mast cells of the thymus capsule, appeared even greenish-black. On rabbit tissues, nearly all mast cells stained pink. Mast cell counts were made on 20 optical fields under objective 40 and ocular 7; this corresponds to a total area of approximately 3.5 mm^2. When the distribution of the mast cells was very irregular, or cells laden with soot or hemosiderin rendered counting difficult, the counts were repeated several times. Pink lumps in the rabbit kidney were counted as full cells, even though they possibly were cell fragments.

TABLE I. Heparin Content and Mast Cell Count of Rat Organs.

Organ	No. of rats*	Mean heparin content: No. of measurements	Mean heparin content: Units/g	Mean mast cell count, No./3.5 mm^2
Liver	19;20	19	1.73± .12‡	10.8±1.6‡
Spleen	20;19	8†	4.84± .35	3.6±1.4
Intestine	20;20	18	5.55± .58	12.5±4.2
Lung	20;19	9†	6.32±1.04	17.2±1.7
Kidney	18;19	15†	12.84±1.69	3.5± .9
Thymus	19;16	2†	13.3	66.2±9.5

* First figure, heparin assay; second figure, mast cell count.

† The smaller organs of 2 to 4, and, in the case of thymus, of 9 and 10 animals, were pooled for heparin assay; the stand. error was computed for No. of measurements indicated.

‡ ± stand. error.

TABLE II. Heparin Content and Mast Cell Count of Rabbit Organs.

Organ	No. of rabbits*	Mean heparin content, units/g	Mean mast cell count, No./3.5 mm^2
Liver	12;11	1.11 ± .14†	28.7 ± 4.4†
Thymus	10;12	1.34 ± .23	21.8 ± 3.8
Intestine	12;12	2.80 ± .35	42.3 ± 3.0
Spleen	11;11	3.50 ± .49	25.2 ± 5.4
Lung	12;12	5.01 ± .92	30.9 ± 5.6
Kidney	12;12	5.22 ± .81	27.5 ± 4.5

* First figure, heparin assay; second figure, mast cell count.

† ± stand. error.

Results. Table I summarizes the results of heparin and mast cell determinations on rat organs. Thymus and kidney showed the highest, and liver the lowest heparin contents, of the tissues examined. The latter finding was unexpected, for in other species, *e.g.*, the dog, the liver is very rich in heparin, and the anticoagulant was first isolated from, and named for this organ. Intestine, lung and spleen contained moderate amounts of heparin. Variations among individual animals were greatest in the case of the kidney and lung.

The observation on rat mast cells agreed with the reports of earlier workers(6,7). The cells varied in size, and many were very large. On liver sections, mast cells aggregated around a few portal veins but were scarce in the main mass of stroma. The high mast cell content of the thymus and the surrounding fat and mesothelia was associated with signs

of thymus involution. In the lung, healthy areas contained a moderate number of mast cells, and portions, where the alveoli were partially closed by proliferating cells, very few. In the intestine the regional differences in mast cell number were very great; as stated by other workers the intestinal mast cells have polymorphous nuclei like basophilic leucocytes. No mast cells were seen in 4 kidney and 11 spleen samples (out of 19).

Heparin contents and mast cell numbers showed a similar distribution pattern only for some of the rat organs tested, *i.e.*, thymus, lung and intestine. In the liver, on the other hand, an intermediate count was associated with a low heparin level. Kidney and spleen showed practically identical mast cell numbers (the lowest of the group), though the former contained about 2½ times as much heparin as the latter.

In the rabbit, the kidney and lung showed the greatest, and liver and thymus the lowest heparin contents of the tissues examined. In all cases, the values were lower than those of the corresponding rat organs, but the pattern of distribution over the different organs was similar in these two species, with exception of the thymus. Regional variations in heparin content were observed when portions of the same lung or kidney were assayed separately.

The mast cell counts of the different rabbit organs again did not go parallel with their heparin contents. The distribution of cells was more even than in the rat; rabbit tissues exceeded the corresponding rat organs in mast cell numbers, excepting the thymus, but the cells took on only a pale color and were of moderate size. The low number of thymic mast cells might be related to the mildness of involutionary processes.

Discussion. The authors are aware of the difficulties involved in a comparison of tissue heparin studies of different laboratories, since almost every investigator uses different procedures, or, at least, different modifications of certain technics, for extraction and assay of the anticoagulant. Only a few papers in the literature deal with quantitative determinations of tissue heparin levels in rats and rabbits. Wilander and Jorpes observed rat liver to be free of heparin(2), a result not much out of line with the low value found in the present investigation, but indicating that the new method used by the present authors may be more sensitive. Recently, Monkhouse reported values for "extractable heparin" in several rabbit organs significantly lower (about 5 to 100 times) than those presented above(8). This discrepancy might be explained, in part, on the basis of differences in rabbit strain and in assay procedure; the possibility is also considered, however, that the extraction procedure used in the present work is more complete.

The data on the mast cell content of rat organs presented above are in line with results of other workers(6,7). Statements in the literature dealing with rabbit mast cells vary widely. In the present work, relatively high mast cell numbers were observed in rabbit tissues, in agreement with findings of Holmgren and Wilander(1). Michels(9), Constantinides(4), and most earlier authors, on the other hand, reported low mast cell counts in this species. In part, this discrepancy might be explained on the basis of differences in strain and age of the animals. Furthermore. it is conceivable that the fixation was inadequate in some of the early work, since the rabbit mast cell granula are highly soluble.

On the basis of observations by Holmgren and Wilander, Jorpes stated that the stainable material of mast cell granula consists of heparin and that tissue mast cell and "sulphuric ester" content go parallel, the latter being identified with heparin(2). However, when the relationship between tissue heparin and mast cells is studied, not only the cell number, but also the heparin content of the individual cell have to be taken into consideration. Rat mast cells are larger and stain more deeply than those of the rabbit; therefore, individual rat cells contain correspondingly more metachromatic material. These observations might explain why, in the present work, rat tissues yielded more heparin than those of rabbits, although they contained less mast cells. But even when organs of the same species were compared, tissue heparin

and mast cell distributions did not follow the same patterns. If it is assumed that heparin is derived from mast cells, then our results indicate that the distribution of heparin through the body was modified by regional differences, either in the composition of the mast cell granula (proportion of heparin to hyaluronic acid and other mucopolysaccharides), or in the capacity of tissues to retain heparin after its release from the cells.

In the course of the present study, more heparin was found in the tissues of the rat than in the tissues of the rabbit. As the former species exhibits greater resistance to hyperlipemia and atherosclerosis, the observations agree with the theory that the heparin content of the tissues may be one of the factors which contribute to this resistance.

Summary. Tissue heparin contents and mast cell counts were determined on liver, lung, intestine, kidney, spleen and thymus of male rats and rabbits. In most instances, distribution of heparin and mast cells did not follow the same pattern. Rat organs showed higher heparin values, but lower mast cell numbers than the corresponding rabbit tissues.

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Physiological Response of "Insulinase" and Its Inhibitor in the Hypoinsulin State.* (22906)

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Mirsky *et al.*(1-6) have demonstrated a tissue activity in liver capable of inactivating insulin and have named this activity "Insulinase". They also have described the ability of filtrates from heated homogenates of the same livers to inhibit Insulinase. The response of this system of Insulinase and its inhibitor to the level of circulating insulin and its conceivable role in the homeostasis of blood sugar values in the frog and rat have been investigated.

Methods. Intact and depancreatized frogs (*Rana pipiens*) were divided into 2 groups: one group maintained at 23°C and the other at 2-5°C. Pancreatectomy in frogs was accomplished as follows. The frogs were anesthetized with ether and a mid-line abdominal incision was made exposing the pancreas. The major blood vessels of this organ were ligated and cut prior to removal of the pancreas, and the abdomen was then closed in the usual manner. 5-0 silk was used throughout the procedure. The following day the group of normal and operated animals kept at 23°C were force-fed (minced calves liver and bone meal dipped in cod liver oil). After 6-7 days, frogs of both groups were pithed, blood samples were taken by heart puncture for sugar determinations(7), and the livers then extirpated, chilled in ice-water and prepared as indicated below. Male rats of the Holtzman strain weighing 175-200 g were kept in individual wire-bottomed cages and fed a stock diet. Animals were fasted for 65 hours prior to subcutaneous injection of 15 mg of alloxan (Eastman) per 100 g body weight as a 3%

* The authors wish to acknowledge the assistance of Lois I. Prirster for nitrogen determinations.

solution, and subsequently kept on the mixed diet for 15 days. At this time they were transferred to metabolism cages and supplied with 10% sucrose in the drinking water as sole source of calories. Urine was collected for 18 hours and the glucose excretion determined(7) and recorded in Table III. The animals were grouped according to severity of their diabetes as judged from their glycosuria, and the livers of each group were pooled for assays. The blotted livers from rats or frogs were weighed and homogenized in Waring blender for 2 minutes with 4 volumes of M/15 phosphate buffer of pH 7.8. The homogenate was filtered through gauze and cotton and centrifuged at 2,000 r.p.m. for 20-30 minutes. The supernatant was adjusted to pH 7.8, if necessary, and again filtered. This supernatant was either used immediately or kept frozen at –20°C until assayed. Previous experiments have established that Insulinase and its inhibitor were stable at –20°C for periods of at least 4 months. *"Insulinase inhibitor"* was obtained from aliquot of the same supernatant by boiling for 5 minutes, filtering through gauze-cotton pad and adjusting to pH 7.8. Total nitrogen and non-protein nitrogen were determined on all fractions and dilutions of about equal nitrogen content were used for the assays. Assays for *Insulinase* and its inhibitor were essentially similar to those of Mirsky *et al.*(1-6). The substrate was crystallized insulin, (20-23 units /mg), containing approximately 1/1000th of its weight of I^{131} treated insulin. Contrary to Tomizawa *et al.*(8), no difference was observed in rate or extent of Insulinase action at substrate level of 50-800 γ/ml, if the preparation contained one part of iodinated insulin in any dilution from 1 to 100,000 parts of normal insulin. A typical example using 200 γ insulin substrate, shaken with 1 ml of standard rat liver Insulinase for 30 minutes at 38°C in a Dubnoff water bath, is illustrated in Table I. To 0.5 ml of insulin substrate (400 γ/ml of Sørensen's buffer) Insulinase and/or its inhibitor was added in 0.2, 0.5 or 1 ml quantities, and volumes were adjusted to 2 ml with Sørensen's M/15 pH 7.8 phosphate buffer. The mixture was incubated at 38°C for periods of 30 minutes to 2 hours in a Dubnoff water bath with shaking at 100 oscillations per minute. After incubation, the reaction was stopped with 2 ml 10% trichloroacetic acid and one hour later centrifuged for 10-20 minutes. The radioactivity of the whole mixture and of the TCA supernatant was

TABLE I. Hydrolysis of Insulin.

Insulin I^{131} : Insulin	% hydrolysis by stand. "Insulinase"
1 : 1	25
1 : 3	21
1 : 600	24
1 : 6,000	28
1 : 12,000	26
1 : 123,000	25

TABLE II. Normal and Depancreatized Frogs.

	Blood glucose, mg %*	Insulinase hydrolysis of insulin after 2 hr	Heated Insulinase: Hydrolysis of insulin after 2 hr	Heated Insulinase: Inhibition of homologous unheated Insulinase
		%		
Hibernated (2–5°C)				
Depancreatized	3– 27	17–18	3	50
Normals	0	16–18	0	50
Active (20–22°C)				
Depancreatized	83–124	24–27	0	18–57
Normals	0– 20	22–27	4	15–26

* Blood sugars were unusually low in all cases though there is a significant difference between normals and depancreatized animals. In the case of the hibernating animal this is probably merely a reflection of the decreased metabolic state. Depancreatized animals, both hibernated and active, were obviously ill but hibernated animals appeared to fare worse than active animals. It may be that in hibernation even a small increase in blood sugar may be physiologically untenable in relation to other decreased metabolic functions.

TABLE III. Normal and Alloxanized Rats.

A		B	Heated Insulinase C	D
Avg glucose excreted/18 hr, mg	No. of animals	Insulinase hydrolysis of insulin after 2 hr	Hydrolysis of insulin after 2 hr	Inhibition of homologous unheated Insulinase
		%		
21.8 (Controls)	5	36 to 51	25	17
120.5 (Mildly diabetic)	3	34 to 56	26	9
690 (" ")	2	26 to 45	23	16
2,235 (Diabetic)	2	33 to 59	36	8
4,845 (")	4	40 to 51	25	13
5,942 (")	4	24 to 45	16	12

measured in a well-type scintillation counter and the counts corrected for background. Percent hydrolysis, as recorded in the Tables, was calculated by dividing the counts per minute per ml in the TCA supernatants by the counts per minute per ml of the whole mixture. Corrections were made for the small amount of radioactivity found in the TCA supernatants prepared at zero time. In all assays a "standard Insulinase" preparation from rat liver was run simultaneously to assure proper functioning of the assay technic.

Results. The results obtained are summarized in Tables II and III and the variations indicated in the Tables refer to results obtained in 3 different experiments on the same samples.

From the data it is apparent that there is no significant influence of the availability of insulin on either Insulinase or "Insulinase inhibitor". Under the conditions of these experiments the Insulinase system could not have influenced the blood sugar balance by an effect on either internally or externally provided insulin.

The data also shed some light on the nature of the Insulinase system. From columns C & D in Table III, it is apparent that there is substantial hydrolysis of insulin by heated Insulinase preparations. This has been observed by us regularly in rat and beef livers. We were also unable to obtain a graded response to varying amounts of Insulinase preparation using a wide variety of substrate concentrations (50 to 1600 γ) or incubation periods (15 minutes to 4 hours). Extensive separation and purification of the various enzymatic and non-enzymatic entities involved in the degradation of insulin by liver will be necessary to clarify the nature of the observed effects.

Summary. No evidence has been found that the "Insulinase"-"Insulinase inhibitor" system responds *in vivo* (rat and frog) to the absence of circulating insulin.

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Mechanism of Intrinsic Factor Action in the Gastrectomized Rat.* (22907)

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It has recently been demonstrated that the gastrectomized rat is unable to absorb vit. B_{12}(1-4). This defect can be abolished by simultaneous administration of rat gastric juice(3,4) or of preparations containing the glandular portion of the rat stomach(2,4). The disturbance in vit. B_{12} absorption in gastrectomized rats therefore is similar to that which exists in patients with pernicious anemia(5,6,7) or in patients in whom a total gastrectomy has been performed(8). However, although human gastric juice or preparations of hog stomach with intrinsic factor activity are capable of enhancing the absorption of Co^{60}–B_{12} in pernicious anemia and in patients following total gastrectomy, these substances are not effective in the gastrectomized rat(1-4).

A number of theories have been proposed to explain the action of intrinsic factor. It has been suggested that it binds vit. B_{12}(9), thereby either "activating" it(10) or making it unavailable to micro-organisms(11,12) or parasites(13,14) present in the intestine. According to a different hypothesis(15) the intrinsic factor acts primarily on the intestinal wall and in some way specifically facilitates absorption of vit. B_{12}. The purpose of our experiments was to determine whether the more significant relation was between intrinsic factor and vit. B_{12} or between intrinsic factor and the intestinal wall.

Methods. Two different technics were used in securing data about the effects of rat intrinsic factor upon Co^{60}–B_{12} absorption in the gastrectomized rat. The first method employed totally gastrectomized rats; and the experimental procedures, which involved determinations of radioactivity of the feces following a single administration of a test dose of Co^{60}–B_{12}, were essentially similar to those previously reported(4). The second method involved experiments designed to determine the radioactivity present in the wall of isolated loops of small intestine created at a second operation in rats that had previously been subjected to total gastrectomy. In these experiments, a test dose of Co^{60}–B_{12} was perfused in the presence of intrinsic factor or of a control substance at an established rate for a specified time through each of a pair of intestinal loops in the same animal. The loops were then washed briefly, removed and measured for radioactivity. The radioactive Co^{60}–B_{12}‡ employed had a specific activity of 1.08 $\mu c/\mu g$ and was given, unless otherwise specified below, in a test dose of 0.015 μg dissolved in 1 ml of water.

A. *Experiments with gastrectomized rats.* After total gastrectomy, animals were fed a vit. B_{12}-free, low residue diet containing 18% casein as the source of protein. Gastrectomized rats were not used in experiments until at least 10 days after operation and only if they appeared to be in good health. At least 5 days elapsed between successive administrations of Co^{60}–B_{12} in the same gastrectomized animal. During the experiments the animals were kept in individual metabolism cages permitting total collection of stools without coprophagy, during a 4-day period following administration of the test dose of Co^{60}–B_{12}, simultaneously with or without substances to be tested for intrinsic factor activity. These were prepared in liquid form and were injected from different syringes in measured amounts by means of a small rubber catheter about 3 mm in diameter, that had been cautiously inserted through the esophagus for a distance of 8-10 cm. The animals had no

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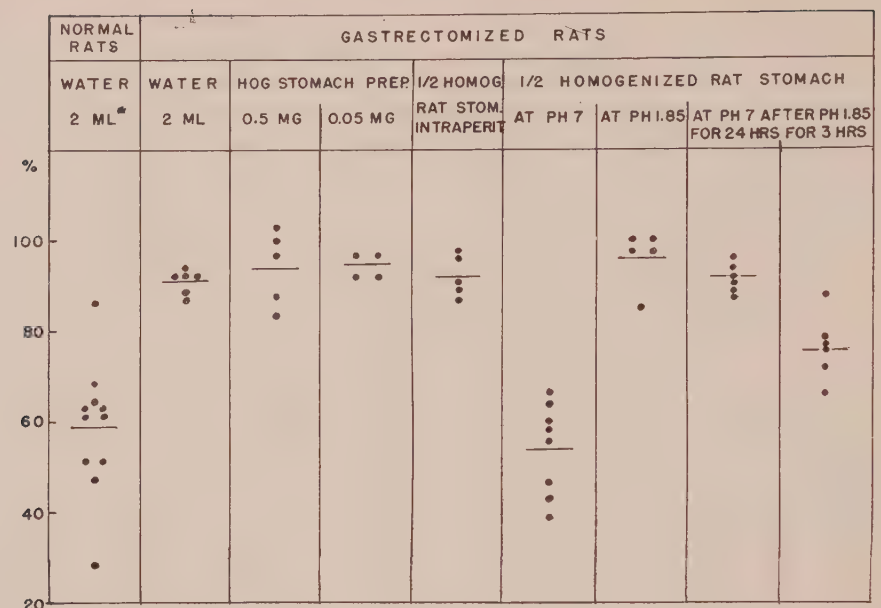

FIG. 1. Radioactivity recovered in feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated. Each dot represents the result of an experiment on a single rat; bars indicate averages.

* Data from previous observations(4).

access to food for at least 3 hours before and 3 hours after administration of test materials.

A highly purified preparation of hog intrinsic factor, WES 386,§ was administered simultaneously with Co^{60}–B_{12} to determine whether, in the virtual absence of interfering substances present in cruder preparations of hog intrinsic factor(16), the latter would be effective like rat intrinsic factor. Five mg of this preparation were considered to possess the intrinsic factor activity present in 1 oral unit of a pharmaceutical preparation of vit. B_{12} with Intrinsic Factor Concentrate, U.S.P. In a first group of gastrectomized rats, the test dose of Co^{60}–B_{12} alone was administered in 2 ml of water. In a second group it was given with 0.5 mg of WES 386 in 2 ml of water. In a third group the dose of WES 386 was 0.05 mg in 2 ml of water. From Fig. 1 it can be seen that this hog stomach preparation was without detectable effect upon vit. B_{12} absorption at either dosage level.

§ Kindly made available to us by Dr. W. L. Williams of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

An intraperitoneal injection of ½ a rat stomach homogenized in 5 ml of water was given to each of a group of gastrectomized rats. Simultaneously, each animal was given the test dose of Co^{60}–B_{12} by stomach tube. As shown in Fig. 1, there was no enhancement of absorption of radioactive vit. B_{12} although this readily occurs when these substances are given together by mouth in such amounts(4).

Homogenized rat stomach was administered at pH 1.85 and at neutrality to determine the effect of maintained acidity upon the action of rat intrinsic factor because of the results of previous observations(17) of similar type in pernicious anemia. The buffering effect of the stomach tissue was such that in order to lower the pH to 1.85, it was necessary to add about 4 ml normal HCl to the test dose of ½ of a homogenized rat stomach, about 0.75 g. The suspension was made up to 5 ml with water. In a first group of gastrectomized rats the rat stomach preparation was given at pH 7 simultaneously with the test dose of Co^{60}–B_{12}. In a second group the rat stomach was given at pH 1.85. In a third group, fol-

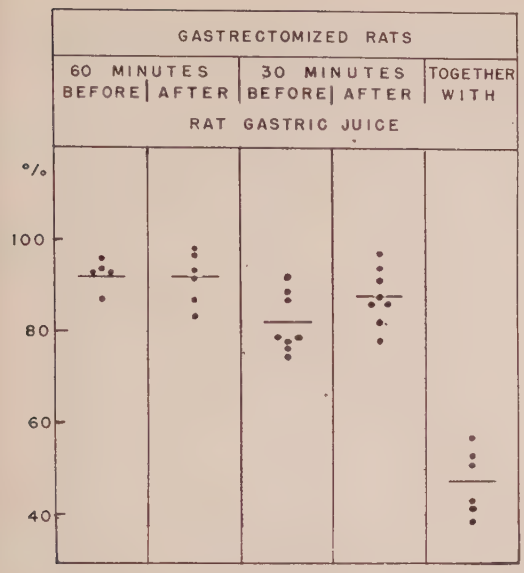

FIG. 2. Radioactivity recovered in feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated. Each dot represents the result of an experiment on a single rat; bars indicate averages.

lowing incubation at pH 1.85 for 24 hours at 37.5°C, the rat stomach was given at pH 7 immediately after addition of NaOH. In a fourth group, following incubation at pH 1.85 for 3 hours at 37.5°C, the homogenized rat stomach after similar treatment with NaOH was given at pH 7. The results shown in Fig. 1 indicate that acidification prevented the function of rat intrinsic factor *in vivo*.

Influence of the order of administration of Co^{60}–B_{12} and rat gastric juice when separated by an interval was studied because of the results of previous observations(15) of similar type in pernicious anemia. A first group of gastrectomized rats was given the test dose of Co^{60}–B_{12}, dissolved in 3 ml of water, and this was followed after 60 minutes by 3 ml of rat gastric juice. A second group of rats received the Co^{60}–B_{12} 60 minutes after the rat gastric juice. In a third group, the Co^{60}–B_{12} preceded the rat gastric juice by 30 minutes; and in a fourth group rat gastric juice preceded the Co^{60}–B_{12} by 30 minutes. As may be seen in Fig. 2, there was no definite evidence of difference due to the order of administration of the 2 substances. However, when Co^{60}–B_{12} and rat gastric juice were given simultaneously to a final group of rats the usual effect of rat intrinsic factor was manifest by decreased excretion of radioactivity in the feces.

B. *Experiments with isolated loops of small intestine with intact blood supply.* Gastrectomized rats were fasted 24 hours and then anesthetized by an intraperitoneal injection of Nembutal (pentobarbital sodium). A midline incision was made in the anterior abdominal wall and the ileum was ligated about 10-15 cm from the ileocecal junction. A polyethylene catheter about 3 mm in diameter was then introduced through the wall of the gut about 1 cm above the first ligature and tied in place pointing upward. About 10 cm higher up along the intestine another polyethylene catheter was introduced and tied in place pointing downward. Thus an isolated loop of intestine with intact blood supply was created that could be perfused by test mixtures introduced into the intestine through the upper catheter and flowing out through the lower. A second 10 cm loop of intestine was then created slightly higher up in the intestine than the first and was similarly isolated between 2 catheters. Throughout the operative procedure the intestine was handled with care in order to maintain the circulation as evidenced by arterial pulsation and normal color. The abdominal wall was then closed by sutures, with care to prevent obstruction of the intra-intestinal orifices of the catheters or angulation of the gut by malpositioning of catheters as they emerged through the abdominal incision.

In the performance of experiments, both isolated loops were simultaneously perfused through their respective upper catheters with the 2 different mixtures to be tested at a rate of 1 ml for the first minute and of 0.2 ml/min for the next 15 minutes. After this, the loops were at once rinsed out with 6 ml of saline and then were emptied of the saline solution by introduction of air through the upper catheter. The loops were then taken out of the abdomen, the catheters removed and the mesentery trimmed at its intestinal insertion. Each intestinal segment was then put into a previously weighed tube, containing 3 ml of

TABLE I. Radioactivity of Pairs of Perfused Intestinal Loops Expressed as Counts/Min./g Tissue.

Rat No.	A. Co^{60}-B_{12} perfused with: Rat gastric juice	Heated rat gastric juice
	Upper loop	Lower loop
1	801	83
3	915	227
5	1112	119
	Lower loop	Upper loop
2	922	490
4	641	102
Avg	878	204

Rat No.	B. Co^{60}-B_{12} perfused with: Human gastric juice	Heated human gastric juice
	Upper loop	Lower loop
1	393	211
3	270	233
5	189	301
	Lower loop	Upper loop
2	141	219
4	279	221
Avg	254	237

concentrated H_2SO_4. The tubes were then reweighed to determine weight of the tissue by difference, and its dissolution was effected by vigorous shaking. Finally, the amount of radioactivity present in each tube was determined in a well-type scintillation counter for which the background was about 500 counts/min. The results are expressed in Table I as counts/min/g of tissue. To minimize any differences characteristic of upper or lower loops of intestine the test mixture in one rat was perfused through either the upper or the lower isolated loop and in the next rat the same mixture was perfused through either the lower or the upper loop, respectively. However, in none of the experiments were consistent differences between radioactivity of upper and lower intestinal loops observed.

In one group of rats so prepared *a mixture of 2 ml of neutralized rat gastric juice and 2 ml of 25% human albumin,* to which 0.015 μg of Co^{60}–B_{12} had been added, was perfused through one intestinal loop. Through the other loop were perfused 2 ml of neutralized rat gastric juice which had been heated for 10 minutes at 100°C to destroy its intrinsic factor activity(4) and 2 ml of 25% human albumin to which 0.015 μg of Co^{60}–B_{12} had been added. The results shown in Table I indicate that unheated rat gastric juice was distinctly more active in promoting absorption of vit. B_{12} by the intestinal wall than was heated rat gastric juice. In a second group of such rats the experimental procedure was identical except that *neutralized normal human gastric juice* was employed instead of rat gastric juice as a source of intrinsic factor. The results shown in Table I indicate no consistently greater activity of unheated compared to that of heated human gastric juice in promoting absorption of Co^{60}–B_{12}.

In a final set of experiments the effect of *prior in vitro saturation of the binding capacity of rat gastric juice with labelled or with unlabelled vit. B_{12},* respectively, was studied by simultaneous perfusion of a pair of isolated intestinal loops. For example, the first mixture was prepared by adding 0.8 μg of Co^{60}–B_{12} to 20 ml of rat gastric juice which was then dialyzed in a cellophane bag for 24 hours against water in the refrigerator. Radioactivity measurements indicated that only 0.22 μg of labelled vit. B_{12} remained within the bag, presumably in a bound state. It was assumed that similar amounts of unlabelled vit. B_{12} remained in an aliquot of the sample of rat gastric juice treated at the same time in the same way after addition of the same amount of unlabelled vit. B_{12}. The contents of the bags were removed and kept frozen until required for use.

When an experiment was run, to 2 ml of dialyzed rat gastric juice containing labelled vit. B_{12} was added an equal amount of unlabelled vit. B_{12} in 2 ml of saline, together with 0.6 ml of saline. Four ml of this mixture, which was prepared immediately before use, were perfused through one intestinal loop, while the other intestinal loop was perfused with a mixture prepared in identical fashion except that the rat gastric juice had been first exposed to unlabelled vit. B_{12} and subsequently, after dialysis, to labelled vit. B_{12}. Because all unbound vit. B_{12} had presumably been lost from the dialysis bag, it was assumed that the vit. B_{12} subsequently added

TABLE II. Radioactivity of Pairs of Perfused Intestinal Loops Expressed as Counts/Min./g Tissue.

Rat No.	Rat gastric juice first saturated with: Unlabelled vit. B_{12}	Labelled vit. B_{12}
	Upper loop	Lower loop
1	108	476
3	468	1368
5	294	587
7	460	1261
8	474	2195
9	862	2209
	Lower loop	Upper loop
2	538	420
4	574	750
6	289	927
Avg	451	1132

could not immediately replace that present in the bound form. Consequently, the experimental design permitted one intestinal loop to be perfused with rat gastric juice in which all or most of the bound vit. B_{12} was radioactive while the other loop was perfused with rat gastric juice in which the same amount of bound vit. B_{12} was non-radioactive. The results are shown in Table II. In the experiment on Rat No. 1 the amount of radioactive vit. B_{12} and of non-radioactive vit. B_{12}, respectively, perfused through each of the two loops was 0.022 μg. In experiments on 8 other rats both these values were 0.031 μg. The data in Table II, with one exception, indicate a greater amount of radioactivity in the wall of the intestinal loop when the rat gastric juice had been initially exposed to labelled vit. B_{12} than when the first exposure had been to unlabelled vit. B_{12}.

Discussion. Human gastric juice(4), hog gastric juice(2) and a commercial hog stomach preparation(1) did not increase the absorption of Co^{60}–B_{12} in the gastrectomized rat. On the other hand, rat stomach preparations(2-4) and rat gastric juice(3,4) were active in this respect. In normal rats with isolated loops of small intestine with intact blood supply during 20-hour periods the absorption of Co^{57}–B_{12} was enhanced by preparations of rat stomach and inhibited by preparations of hog duodenum(18). This apparent species specificity of intrinsic factor was also demonstrated in the present short term experiments with paired isolated loops of small bowel with intact blood supply created in previously gastrectomized, anesthetized rats. As shown in Table I the radioactivity of the wall of the intestinal loop perfused for 16 minutes with Co^{60}–B_{12} and rat gastric juice was consistently greater than that of the paired loop perfused with Co^{60}–B_{12} and rat gastric juice previously heated to 100°C for 10 minutes to destroy intrinsic factor activity(4,19). On the other hand, no consistent difference was observed with heated and unheated human gastric juice. Maintenance of the circulation is seemingly important for the function of intrinsic factor because in experiments(20) with segments of rat intestine removed from the animal, rat gastric juice has been shown to inhibit the uptake of radioactive vit. B_{12} by the tissue.

Because relatively crude preparations of hog intrinsic factor may antagonize the absorption of Co^{60}–B_{12} in normal rats(1,21) and human subjects(16), the most highly purified preparation of hog intrinsic factor available to us was tested. As shown in Fig. 1, this preparation, WES 386, in test doses of 0.5 and 0.05 mg, respectively, equivalent to 1/10 and 1/100 of a U.S.P. unit of intrinsic factor activity, and containing less than 0.05 $\mu\mu$g and 0.005 $\mu\mu$g of unlabelled vit. B_{12}, was without effect on the absorption of 0.015 μg of Co^{60}–B_{12} in the gastrectomized rat. Consequently, the species specificity of rat intrinsic factor suggested by others(1) now appears to have been demonstrated and presumably explains the similar inactivity of human gastric juice(4) in the gastrectomized rat. It is of interest, however, that the difference between the activity of human and of hog intrinsic factor in pernicious anemia (22-23) is but slight.

Although several kinds of tissue proteins bind vit. B_{12}(24), extracts of hog stomach mucosa bind relatively large amounts(25) as do highly purified and clinically active preparations of hog intrinsic factor(26,27). Moreover, electrophoretic dispersions of normal human gastric juice exhibit several peaks of binding activity(28) which are reduced by exposure to pH 10 to one component which

alone possesses intrinsic factor activity(29). As shown here rat gastric juice also binds vit. B_{12}. It is therefore possible that binding capacity is a characteristic common to all species of intrinsic factor. According to the results of 9 experiments shown in Table II, with 1 exception, the absorption of Co^{60}–B_{12} was greater when the labelled rather than the unlabelled form of vit. B_{12} was "bound" by the rat gastric juice. This result, which confirms similar observations(30) in pernicious anemia, clearly suggests that binding of vit. B_{12} to intrinsic factor is of importance in its absorption. Recent observations(31) employing rat and hog stomach preparations have likewise emphasized the positive influence of priority of contact with Co^{60}–B_{12} upon its assimilation. However, if vit. B_{12} binding is a property common to the intrinsic factor of different species, it cannot in itself be responsible for the marked specificity of rat gastric intrinsic factor for the rat; and some additional property of rat intrinsic factor must be decisive in determining the ready assimilation of Co^{60}–B_{12} by the gastrectomized rat. Moreover, in view of the observation that crude preparations of hog intrinsic factor enhance the absorption of Co^{60}–B_{12} in pernicious anemia but inhibit it in normal subjects(16) as well as in normal rats(21); and that in chickens the absorption of Co^{60}–B_{12} is inhibited by a diet rich in casein (32), the ability of a substance to bind vit. B_{12} seems by itself actually to oppose the normal process of vit. B_{12} assimilation. Therefore, some other facet of the intrinsic factor molecule must be concerned, and may be conceivably involved in a species-specific relation to the intestinal wall.

Consistent with this supposition is the clinical evidence(15) that the hematopoietic activity of vit. B_{12} is greater in pernicious anemia when the administration of intrinsic factor precedes rather than follows it by an interval of a few hours. In such observations the theoretical opportunity for contact between intrinsic factor and vit. B_{12} should be similar irrespective of the order of their administration. On the other hand, a preparatory effect of intrinsic factor on the intestinal wall would be favored by the prior administration of the intrinsic factor. However, as shown in Fig. 2, when a repetition of such clinical observations was attempted in the gastrectomized rat, even with an interval as short as 30 minutes, no certain differences could be ascribed to the order of administration. Indeed, intrinsic factor activity was detected only with simultaneous administration. This negative result does, however, indicate that in the gastrectomized rat the effect of intrinsic factor is a very transient one. Together with the present experiments with "bound" vit. B_{12}, labelled and unlabelled, it suggests that binding between vit. B_{12} and intrinsic factor is required for its enhancement of the absorption of vit. B_{12}.

In the present experiments with gastrectomized rats it was shown (Fig. 1) that rat stomach preparations given at pH 1.85 were devoid of intrinsic factor activity. However, intrinsic factor activity was present after exposure to pH 1.85 for 3 hours *in vitro,* provided the preparation was neutralized thereafter at the time of administration with Co^{60}–B_{12} to the rat. Because an increase of Co^{60}–B_{12} in the livers of intact rats has been observed to take place within 15 minutes of the administration of Co^{60}–B_{12}(20), 3 hours should have provided ample time for the action of intrinsic factor to become manifest unless acidity was in some way inhibitory to an action of intrinsic factor either within the lumen of or on the surface of the intestine. As the binding capacity of hog stomach mucosal extract for vit. B_{12} is not significantly altered between pH 8 and pH 2(25), and as this also obtains in our experience with rat gastric juice, the inhibitory effect of acidity on the action of intrinsic factor *in vivo* is probably exerted on some other property of intrinsic factor than its binding capacity. Taken together these facts suggest that in addition to binding vit. B_{12} the peculiar activity of intrinsic factor in enhancing the absorption of vit. B_{12} is dependent upon an acid-sensitive relation between intrinsic factor and the intestinal surface.

Summary and conclusions. 1. *In experiments with gastrectomized rats*: (a) The

probable species specificity of rat intrinsic factor was demonstrated by the failure of a highly purified preparation of hog intrinsic factor to increase the absorption of Co^{60}–B_{12}. (b) In consonance with previous observations in pernicious anemia, acidification (pH 1.85) of a mixture of Co^{60}–B_{12} and homogenized rat stomach inhibited the absorption of Co^{60}–B_{12}. (c) In contrast to previous observations in pernicious anemia, separate serial administration of Co^{60}–B_{12} and rat gastric juice failed to show that rat intrinsic factor was more effective in promoting the absorption of Co^{60}–B_{12} when it preceded than when it followed the Co^{60}–B_{12}. Indeed, no certain effect of rat intrinsic factor was detected whether it preceded or followed the Co^{60}–B_{12} by as short an interval as 30 minutes. 2. *In experiments with paired isolated loops of small bowel with intact blood supply created in previously gastrectomized rats*: (a) The radioactivity of the wall of the loop perfused with Co^{60}–B_{12} and rat gastric juice was greater than that of the paired loop perfused with Co^{60}–B_{12} and rat gastric juice previously heated to destroy its intrinsic factor activity. No consistent difference was observed with heated or unheated human gastric juice. (b) The absorption of Co^{60}–B_{12} was greater when labelled rather than unlabelled vit. B_{12} in equal amounts had saturated the binding capacity of rat gastric juice before admixture with an equal amount of the other form of vit. B_{12} just before the intestinal perfusion. 3. *In conclusion,* binding of vit. B_{12} is an important, perhaps indispensable, aspect of the function of intrinsic factor. However, the fact that vit. B_{12} binding is also characteristic of gastric preparations of man and of hog, which are without intrinsic factor activity in the rat, suggests that some property of intrinsic factor in addition to its vit. B_{12} binding capacity is required for intrinsic factor activity. Possibly this property concerns a relation between intrinsic factor and the surface of the intestinal mucosa which is disturbed when experimentally the contents of the alimentary tract are made strongly acid.

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Requirement for Properdin in Hemolysis of Human Erythrocytes Treated with Tannic Acid.* (22908)

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The hemolysis of the abnormal erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) by normal human serum requires the properdin system, which consists of properdin, the components of complement, and magnesium(1). Normal human erythrocytes treated with tannic acid (TA cells) are also hemolysed by normal serum(2). The present report indicates that the hemolysis of appropriately treated TA cells also requires the properdin system.

Methods. Erythrocytes. Human group O blood was collected in an equal volume of Alsever's solution and stored at 4°C. The blood was stored 4 days before use and discarded after 10 days. The same donor was used throughout, although blood samples from other donors were equally satisfactory. The erythrocytes were washed 3 times in 0.15 M NaCl and suspended to 2% in barbital buffer containing 0.0005 M $MgCl_2$(3,4). *Tanning of cells.* A stock solution of 1% tannic acid in 0.15 M NaCl was stored at 4°C. It was diluted immediately before use in 0.15 M NaCl to concentrations optimal for tanning cells. The final concentration of tannic acid employed was usually 1:15,000. There was some variation from day to day, and concentrations from 1:10,000 to 1:80,000 were tested each day. The activity of the properdin system was demonstrated only for cells exposed to a relatively limited range of concentrations of tannic acid. Concentrations of greater than 1:10,000 caused clumping of erythrocytes, slight spontaneous hemolysis, and marked hemolysis in serum lacking properdin (RP). Concentrations of less than 1:20,000 usually failed to alter the cells. There was little difference in activity whether the tanning was carried out in silicone treated or ordinary glassware. Aliquots of 30 ml of erythrocyte suspension and tannic acid solution of optimal concentration, in separate 150 ml Erlenmeyer flasks, were placed in a 37°C water bath until the contents of each flask reached a temperature of at least 36°C. The tannic acid solution was then poured rapidly into the cell suspension with continuous and vigorous swirling for 5 to 10 seconds. The mixture was immediately transferred to test tubes and centrifuged for 1 minute at approxi-

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TABLE I. Hemolysis of Erythrocytes Treated with Tannic Acid.

Reagent	Hemolysis	
	Without added properdin	With added properdin (10 u)
Human serum	+	+
Serum heated to 56°C	0	0
RP	0	+
R 1	0	0
R 2	±	+
R 3	0	0
R 4	0	0
Resin treated serum	0	0
Idem + Ca^{++}	0	0
" + Mg^{++}	+	+

mately 2000 r.p.m.; the cells were well packed. The supernatant fluid was decanted and the packed cells were washed once with a large volume of 0.15 M NaCl; they were centrifuged again at 2000 r.p.m. for 1 minute, and finally suspended to exactly 2% in barbital buffer. The cells were prepared daily, and kept at room temperature during the day of use. Cooling to 4°C and then rewarming resulted in spontaneous hemolysis. *Temperatures* lower than 37°C and periods of exposure to tannic acid of greater than 5 to 10 seconds resulted in inadequate sensitization of the cells. Cells were considered satisfactorily tanned when they were not hemolyzed in RP, or purified properdin, but were hemolyzed by fresh serum or RP to which properdin was added. *Test system.* To 0.25 ml of serum or serum reagent were added varying amounts of properdin, barbital buffer, and 0.05 ml of 2% TA cells, to give a final total volume of 0.50 ml. The mixture was incubated at 37°C for 30 minutes, the tubes centrifuged, and the amount of hemolysis determined in a photoelectric colorimeter. *Serum reagents.* Normal human serum, serum lacking properdin (RP), serum lacking specifically one of the components of complement (R1, R2, R3, R4), and purified properdin, were collected and prepared as previously described(4,5). Serum was treated with amberlite IRC-50 sodium cycle resin to remove Mg^{++} and Ca^{++} as previously described; it was tested immediately(6).

Results. Hemolysis of TA cells in serum did not occur at 4° or 18°, and was optimum at 37°C. Hemolysis was greater at pH 7.4 than at pH 6.8 or 8.0. Therefore, the reaction was allowed to proceed at 37°C and pH 7.4. *Requirement for properdin.* Although marked hemolysis of TA cells occurred when they were incubated at 37°C in normal human serum, serum from which properdin had been removed (RP) failed to hemolyze TA cells (Table I). Addition of 10 units of purified properdin per ml of RP restored the hemolytic activity of the serum. Properdin alone was not hemolytic. Addition of varying amounts of properdin to RP caused a regular increase in hemolysis of TA cells (Fig. 1) proportional to the amount of properdin added. The RP employed in the experiment illustrated was slightly lytic. Properdin alone is not lytic. Addition of small volumes of various human sera to RP resulted in varying amounts of hemolysis. There was no correlation between the amount of hemolysis and the properdin level of the serum as determined by the zymosan assay(4). Variations in total complement in the various sera also did not account for the differences which were observed. Thus, while the TA cells are suitable for the assay of purified properdin, they are not suitable for the quantitative measurement of properdin in serum.

Requirement for complement. Serum from which C'1, C'3, and C'4 had been removed, or inactivated failed to hemolyze TA cells (Table I). Addition of 10 units of properdin per ml to these reagents failed to restore hemolysis. Some serums lacking C'2 activity

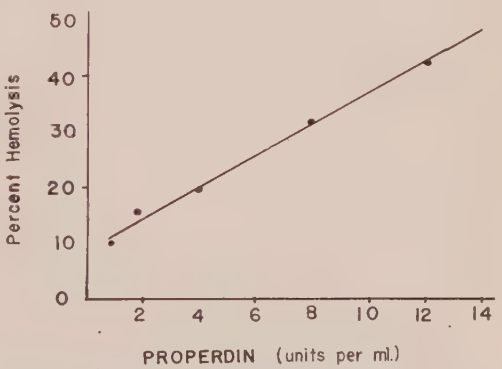

FIG. 1. Effect on hemolysis of tannic acid treated cells, of adding increasing amounts of properdin to RP.

caused slight hemolysis of TA cells which increased upon the addition of 10 units of properdin.

Requirement for Mg^{++}. Serum from which Mg^{++} and Ca^{++} had been removed did not hemolyse TA cells. Addition of 0.0005 M Mg^{++} to the resin-treated serum restored hemolytic activity. Addition of 0.005 M Ca^{++} did not restore hemolytic activity to the resin-treated serum.

Discussion. Under the conditions of the experiments described above, the hemolysis of normal human erythrocytes treated with weak solutions of tannic acid requires properdin, factors indistinguishable from serum complement, and magnesium. In this manner the mechanism of hemolysis of TA cells and PNH erythrocytes by human serum is similar. It differs from the hemolysis by isohemolysins and panhemolysins which require specific antibody and complement, but do not require properdin or magnesium(1).

However, differences exist between TA cells and PNH cells. TA cells spontaneously agglutinate, hemolyze spontaneously following exposure to cold, and lyse optimally in serum at pH 7.4. PNH cells do not spontaneously agglutinate, are stable in the cold, and hemolyze maximally in serum at pH 6.8.

Another slight difference is that C′2 is not an absolute requirement for the hemolysis of TA cells. Thus, the reaction of the properdin system with TA cells resembles its reaction with zymosan and other high molecular weight polysaccharides(7). This suggests that the surface presented by the tanned cell may resemble the polysaccharides which are known to interact with properdin(8).

Although attempts have been made to employ TA cells for the measurement of complement in serum, the methods used differed from those employed in the present study(9). It appears likely that the degree of alteration of the cell surface influences the susceptibility of the cell. Cells exposed to dilutions of tannic acid less than 1:10,000 do not require properdin for hemolysis, whereas cells exposed to 1:15,000 dilution of tannic acid required both complement and properdin. Cells exposed to higher dilutions are not hemolyzed.

The hemolysis of TA cells has not proven reliable as a simplified method for the assay of properdin in human serum. As mentioned above, other as yet undefined factors in normal serum also influence hemolysis. Thus there is no correlation between serum properdin level as measured by the zymosan assay and the ability of a serum to hemolyze TA cells. It is also unlikely that such a system would be suitable for the measurement of properdin in animal sera, since TA cells as well as PNH cells are hemolysed by heterolysins, independent of the properdin system.

Summary. 1) The hemolysis of normal human erythrocytes exposed to appropriate dilutions of tannic acid by human serum, requires the properdin system, which consists of properdin, complement, and magnesium. 2) Similarities and differences are pointed out between the properties and mechanism of hemolysis of tannic-acid-treated cells and erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. 3) While the hemolysis of TA cells is suitable for the assay of purified properdin it is not reliable for the quantitative measurement of properdin in serum.

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Methods Employed for Purification of Streptokinase.* (22909)

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Tillett and Sherry(1) introduced the local use of streptokinase into human therapeutics following the description of a method for its partial purification(2). However, the clinical toxicity of the available preparations has been sufficient to preclude its systemic use in man except under restricted conditions(3).

The present communication describes methods for the further purification of streptokinase, and a further paper(4) reports the assay of these preparations in man for clinical toxicity. Though the purified preparations were much less toxic to man than the currently available preparations, activity/nitrogen ratios did not always parallel clinical toxicity. For this reason, alternative methods of purification are briefly described in addition to that finally adopted.

Materials and methods. The streptokinase assay was based on that of Christensen(5) with the modification that dilutions were made directly into each assay tube with a micro-burette.† This modification allowed the use of more closely spaced dilutions than were employed in the original procedure, and increased both speed and accuracy. Assays were expressed in terms of the Christensen unit(5), each assay being the average of duplicate determinations. If the duplicates were discrepant by more than 10%, assays were repeated until satisfactory agreement was obtained. The reproducibility of the assay method was assured by means of frequent control determinations on the National Institute of Health streptokinase standard. *Nitrogen* determinations were made by a standard micro-Kjeldahl method. In addition, optical density at 260 mμ and 280 mμ was followed with a DU Beckman spectrophotometer. *Starch zone electrophoresis* followed the procedure of Kunkel and Slater(6) except that a wider (20 cm) trough was used. Water, at 4-6°C, was pumped through hollow top and bottom plates to provide extra cooling. The buffer had the following composition: sodium borate 8.5 g, boric acid 0.68 g, water to 1 liter adjusted to pH 8.9. Owing to the fact that the borate ion interacts with the starch, the effective ionic strength could not be calculated. The starch‡ was prepared by washing 1 kg twice with 3 liters of 0.05 M acetic acid at 50°C, washing with 5 liters of 1% saline on a Buchner funnel, and then washing twice with 3 liter amounts of borate buffer. Five-six ml of 0.5-2% streptokinase was used for each electrophoresis run, which was made for 16-24 hours at 300-450 volts and 60-100 milliamps. The block was cut into ½-inch strips, each starch strip being placed in a sintered glass funnel and eluted with successive small volumes of 1% saline, containing 0.01 M phosphate buffer at pH 7.6, to a total of 15 ml. A Raymond-type apparatus(7) was used for electrophoresis-convection studies. Paper electrophoresis was performed with a horizontal type machine, using barbital buffer at pH 8.6 and ionic strength 0.1; amido-black stain was used. Streptodornase was determined by a viscosimetric method(8). *Fractionations* were performed at 2-5°C with aqueous solutions, and at −5 to 0°C with organic solvent-aqueous solutions. Saline refers to 0.155 M sodium chloride solution. Sintered glass filters were of medium porosity, unless otherwise stated. Apparatus was acid cleaned, and fractionation procedures were completed as rapidly as possible to prevent contamination with pyrogens. The starting material for fractionation was a lyophilised partially purified streptokinase-streptodornase concentrate.§

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† Micro-metric Instrument Co., Cleveland, O.

‡ Eimer and Amend, New York City.

§ Bulk Varidase® a commercial streptokinase-streptodornase preparation, prepared by a modified Christensen method(3), and kindly supplied by Mr. Frank Ablondi, Lederle Laboratories, Pearl River, N. Y.

Results. In this and the ensuing article(4) reference is made to 4 methods of streptokinase purification. The different methods were not substantial changes in technic, but usually represented additional steps taken beyond those of the previous procedure. For purposes of correlation between the biochemical purification procedures and the clinical effects of the material(4) the streptokinase preparations are specifically identified as methods 1, 2, 3, and 4. The starting material usually assayed at 85 units/gamma N, though 2 lots were 95 units/gamma N. There were differences between lots obtained in bulk with regard to color, solubility, and the amount of insoluble residue. Despite these physical differences all 6 bulk lots used exhibited similar purification characteristics.

Method 1. The concentrate was taken up in versene®‖ (sodium ethylenediaminotetraacetate)-saline-phosphate, and clarified by sintered glass filtration. The filtrate was equilibrated with excess ether in the cold and then quick-frozen in a dry ice-alcohol mixture. Two hours later it was cautiously melted and the lipid layer centrifuged off. The clear aqueous phase was adjusted to pH 5.3 with 1 M acetate buffer and the streptokinase was precipitated with alcohol to a final concentration of 12.5%. The yield of streptokinase was found in different runs to vary between 33-52% of the original, the purity being 150-220 units/gamma N. Four zone electrophoretic runs performed with this material gave yields of 33-65% and potencies of 340-450 units/gamma N with a total of 1-2 million units in each batch. In these as in all the other preparations used in man, the streptodornase content was negligible, being less than 100 units of streptodornase for every million units of streptokinase. The final chemical purification method is described below.

Method 2. Ten g of streptokinase concentrate, 100 ml of 0.5 M versene and 1600 ml of 0.02 M phosphate-saline were adjusted to pH 9.0 with 0.1 N sodium hydroxide. The mixture was stirred until most of the material had dissolved, the pH was then lowered to 8.0 with 0.1 N hydrochloric acid. (Two batches were largely insoluble at pH 9.0, but dissolved when the pH was raised to 11.0 for 2-3 minutes.) Forty g of washed celite¶ was added, and after 30 minutes of stirring, the mixture was filtered through sintered glass. Four hundred g of calcium phosphate gel(9) was stirred with the filtrate for 1 hour, after which the gel was removed by centrifugation. 10-15% of the nitrogen was removed at this step with some 5% of the streptokinase activity, the filtrate changed from opalescent to clear, and the 260/280 optical density ratio fell from 0.76 to 0.64. The filtrate was brought to 30% ammonium sulphate saturation and after 3 hours was filtered through sintered glass using 5-10 g of celite as a filter aid. The precipitate was discarded, the filtrate brought to 42% ammonium sulphate saturation and left overnight. The precipitate was collected on a 30 cm Buchner funnel using No. 50 Whatman paper, it was washed with 200 ml of saline at 42% ammonium sulphate saturation. The precipitate and paper were eluted with 400 ml of saline containing 0.025 M tris (hydroxymethyl) aminomethane buffer at pH 8.0. The solution was readjusted to pH 8.0 with 0.1 N sodium hydroxide and filtered through sintered glass. The yield at this point was 45-60% of the starting activity, the purity usually 300 units /gamma N and the 260/280 optical density ratio 0.53. The filtrate was cooled to 0°C in a refrigerated tank and ether (precooled to −5°C, 50 ml per 100 ml of filtrate) was added with stirring over a period of 3 hours. The temperature was slowly lowered during this time until, at full ether saturation, it was −5°C. The mixture was centrifuged at −5°C and 400 × g for 1 hour in centrifuge separating funnels. The ether-aqueous layer was again equilibrated with ether (25 ml ether per 100 ml of solution) and centrifuged. The ether-aqueous layer (300-360 ml) was added to 500 ml of ether saturated distilled water containing 4 ml of 0.2 M sodium acetate. The pH was adjusted to 6.2 with 0.2 M acetic acid and sufficient 1 M zinc acetate

‖ Brand of sodium ethylenediaminotetraacetate, Bersworth Chemical Co., Framingham, Mass.

¶ Analytical Celite, Johns-Mansville Products.

added to bring the zinc concentration to 0.01 M. Next morning the solution was centrifuged and the precipitate taken up in 15 ml of 0.25 M versene-saline. (The yield was 13-19 million units representing 15-20% of the original activity, the purity 370-430 units/gamma N and the 260/280 optical density ratio 0.52.)

Method 3. One batch of method 2 material was precipitated with 42% ammonium sulphate, dialyzed, and precipitated with zinc at 0.01 M concentration. Nearly half the streptokinase activity was lost at this step and the potency was only raised from 370 to 410 units/gamma N. However, in view of its different toxicity to man, this is referred to as *method 3.*

Method 4. Three lots of method 2 material were subjected to starch zone electrophoresis and streptokinase assaying at 515, 600 and 600 units/gamma N was obtained with a yield of 35-60%.

Characterization. The fractionation procedures were guided by paper electrophoresis. It was found that streptokinase moved with the mobility of an alpha 2 globulin in barbiturate buffer at pH 8.6. Unfortunately at purities of 400 units/gamma N and above, this procedure failed to provide useful information, as the material frequently ran as one component. Furthermore, material that had been subjected to starch zone electrophoresis sometimes showed more trailing than the original material of lesser potency.

Ultra-centrifuge and classical electrophoresis examination were made upon a sample of method 2 material, assaying at 430 units/gamma N, which ran as one component without detectable trailing on paper electrophoresis. The ultracentrifuge showed two components, the first was a single peak containing 88% of the sample with a sedimentation constant of 3.4 in pH 7.6 phosphate buffer of 0.1 ionic strength. The second component was of broad distribution with an approximate mean constant of 1.2. These figures can be converted to S_{20w} by adding 10%. The estimated molecular weight of the main peak was 50,000, and that of the second 5,000-25,000 with a mean of 15,000. Classical electrophoresis at pH 7.4 and pH 8.6 showed in each case 3 components: a slow component (4.7% of the material), a main peak (86.4% of the material) and a fast component (8.9% of the material). The respective mobilities at pH 8.6 and 10^{-5} cm sec/volts/cm were 1.3, 3.8, and 4.2 for the descending phase, and 2.4, 3.5, and 5.2 for the ascending phase. The streptokinase activity was contained in the main peak. It seems clear from the mobilities that the two minor components should be separated from the main peak on starch zone electrophoresis and, indeed, such a distribution was found. However, it would also appear from the consideration of the relative increase of potency obtained after zone electrophoresis (20-30%), that this method of separation also partly resolves the main peak into two components. Neither the material prepared by chemical fractionation, nor that subjected to electrophoresis were homogenous on immuno-chemical analysis. Examination by the technic of Ouchterlony(10) against rabbit antibody showed 5-7 bands for method 2 material (2 samples) though the antigenic structure was much weaker than that of the original concentrate. Electrophoresis of these two samples (method 4) further weakened the antigenic structure and resulted in the disappearance of two bands. Unfortunately, this extremely sensitive test, under these circumstances, could not be used to provide a quantitative estimate of the impurity present.

Stability. Material for stability testing was brought to 1% human albumin final concentration, sterilized by Seitz filtration (Swinney model) and maintained at refrigerator temperature. It was found that in high concentration (approximately 5×10^5 units/ml) the activity fell about 30% in 6 weeks.

Discussion. A 6-7 fold purification of the original material was achieved by the methods described. Immunochemical analysis showed that the material prepared by zone electrophoresis was impure. It is difficult to estimate the amount of impurity as the results of ultra-centrifuge and classical electrophoretic studies suggested the presence of approximately 13% of impurity before zone electrophoresis, yet zone electrophoresis usu-

ally gave a 20-30% increase in activity/nitrogen ratios. It is possible that mildly denatured material may have similar physical and chemical properties to the active substance. This conclusion was supported by the observation that if the biological activity of the material was allowed to decay at room temperature under sterile conditions its electrophoretic and chemical properties were apparently unchanged. Severer conditions did, of course, change these properties. Thus the tests of homogeneity used do not certainly distinguish between biologically active and biologically inactive protein. For this reason no estimate can be offered as to the biological potency of the "pure" streptokinase.

The purification methods, themselves, leave something to be desired. Particularly at the ether stage the manipulations are exacting and the most rigid temperature control is mandatory. Moreover, starch zone electrophoresis, with its inherently low capacity, is an unsuitable method for large-scale purification. It was hoped that this latter procedure could be replaced by electrophoresis-convection, but some 25 runs revealed that the only moderately satisfactory mode of operation was at the isoelectric point of streptokinase (pH 4.7-5.0), and here the solubility was so low that only small amounts of material could be handled. Though the present methods appear to be of an interim nature, they have sufficed to prepare enough material for clinical trial(4) and provide leads for better methods.

Summary. Chemical fractionation of a currently available, partially purified streptokinase-streptodornase concentrate yielded a 4-5 fold purification of the streptokinase. The addition of a starch zone electrophoresis to the methods increased the purification to 6-7 fold. Biophysical examination suggested that the protein was largely homogenous, but immunochemical analysis revealed the presence of extraneous antigenic components. Ultracentrifuge examination suggested that streptokinase has a molecular weight of approximately 50,000.

We are indebted to Dr. Rhodes Trautman of Rockefeller Institute for the ultra-centrifugation examination; to Dr. Dan Moore of College of Physicians and Surgeons for electrophoretic examinations; and to Dr. R. P. Wodehouse of Lederle Laboratories for immunochemical analysis.

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Studies on Erythropoiesis. III. Factors Controlling Erythropoietin Production. (22910)

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The existence of a plasma factor, known as erythropoietin, that is capable of modifying red blood cell production is firmly established. In 1955, we(1) described a bioassay for this plasma factor in the normal rat. The procedure used the incorporation of Fe^{59} into newly-formed erythrocytes as index of rate of erythropoiesis. More recently, we demonstrated that the hypophysectomized rat is a more sensitive assay animal than the normal one(2,3).

Further studies on the temporal relationship of hypophysectomy to changes in erythropoiesis and on erythropoietic response of the hypophysectomized animal to erythropoietin are the subjects of this paper. In addition, other sensitive assay systems that can be produced by methods designed to decrease erythropoiesis are described.

Materials and methods. A) *Preparation of plasma*: On 3 consecutive days, rabbits or rats were bled by cardiac puncture, and on each of these days, the amount withdrawn approximated 2% of body weight. On alternate days thereafter, blood amounting to about 1.5% of their weight was withdrawn. After the hematocrit was reduced to 25% or less, more blood was withdrawn and heparinized, and plasma was separated and stored at −17°C. This procedure, which has been described in detail(1), was followed for preparation of plasma that we shall refer to as "anemic plasma." Samples from the same lot of anemic plasma were always used in comparative experiments. Plasma, which was prepared similarly from blood of normal, previously unbled rabbits or rats served as control. B) *Assay procedure*: Rats in each experimental group discussed below were given intravenously three 2-cc doses of anemic plasma on successive days. Control animals received normal plasma or saline according to the same schedule. The rate of erythropoiesis was then determined as follows(2): Fe^{59} citrate (2 to 3 μc) was injected into the tail vein of the animal at 3-6 hours following last injection of plasma. Sixteen hours after injection of iron, a 1-cc sample of blood was taken by cardiac puncture. Activity of Fe^{59} in erythrocytes of the sample was determined by counting in a well-type scintillation counter.* The amount of radioactivity in the entire circulating red cell mass was calculated and expressed as per cent of injected dose of Fe^{59} in peripheral red cells. C) *Preparation of recipient animals*: *Hypophysectomized rats.* Hypophysectomized male Sprague-Dawley rats,† 4 or 8 weeks old and weighing 75-90 or 140-160 g, were maintained on diet consisting of milk, fresh vegetables, and Rockland mouse pellets *ad libitum*. The rats were given 3 injections of plasma, and the iron was introduced 6 hours after last injection. Polycythemia was produced in normal, 3-month-old male Sprague-Dawley rats by intraperitoneal injections of washed homologous erythrocytes suspended in saline. Ordinarily, 8 daily injections of 2 cc of red cells were sufficient to elevate the hematocrit from a normal value of about 45% to one between 70 and 75%. The rats were used for assay the day following last injection of red cells. Hematocrit values remained between 70 and 75% throughout the assay so that additional red cell injections were unnecessary. At conclusion of assay procedure, blood volumes, determined by the radiochromium method of Gray and Sterling(4), were 6% of body weight in the polycythemic rat as compared with 5% in the normal rat. *Hyperoxic rats.* Normal, 2-month-old male Sprague-Dawley rats, weighing between 150 and 200 g, were placed in oxygen tent into which flowed a continuous supply of oxygen, maintaining an

* Nancy Wood Counterlab, Chicago, Ill., Model SC-2L-42.

† Obtained from Hormone Assay Laboratories, Chicago, Ill.

atmosphere of 85-95% O_2.‡ After 7 days, the rats were divided randomly into treatment groups of 5 animals each, and the 3-day regimen of plasma injection was initiated. The experiment was concluded on 10th day that the rats were in the oxygen tent. During the 10-day period, the animals were out of the high O_2 atmosphere only for the brief time necessary for injection. *Fasted rats.* Normal, 2-month-old male Sprague-Dawley rats, weighing 175 g, were deprived of all food, but water was supplied *ad libitum.* Radiochromium blood volume determinations after 5 days of starvation gave values of 5% body weight in the starved animal as compared to 5% in controls. At various intervals, as indicated below, rats in randomly-selected groups were injected with Fe^{59}, and the percentage of radioiron incorporated into red blood cells was determined. Other groups were given anemic or normal plasma for 2 days before injection of iron. The rats were weighed at time of sampling. The fast was continued in all cases until conclusion of the assay. *Rats injected with dinitrophenol.* Male Sprague-Dawley rats, weighing between 375 and 400 g, were injected subcutaneously with 5 mg of 2,4-dinitrophenol in a 10-ml volume at pH 8.5 daily for 3 days. One-half of the group received daily intravenous injections of 2 ml of anemic plasma while the other half received saline. Rats in the control group received 10 ml of saline instead of dinitrophenol and anemic plasma or saline as test material. Fe^{59} was administered 2 hours after last injection, and the % uptake of iron was determined as described above. In many experiments, studies of the effects of anemic plasma on elements of peripheral blood were done on either animals used for measurements of uptake of iron, or upon others that were used solely for hematologic investigations. These studies are presented in Paper No. IV of this series.

Results. A. *Effect of age of hypophysectomized rats upon response to anemic plasma.* The rate of decline of erythropoiesis following hypophysectomy in 4-or-8-week-old rats and the responsiveness to anemic plasma at several intervals following surgery were compared. The younger rats were also studied for a longer time to observe changes that occur during equilibration of red cell mass and red cell production. The results are summarized in Fig. 1 and Table I.

‡ Determined by Beckman O_2 analyzer.

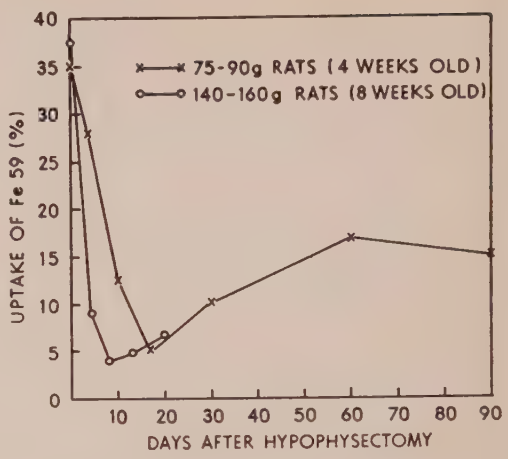

FIG. 1. % incorporation Fe^{59} in erythrocytes of rats hypophysectomized at 4 and 8 wk of age.

In the younger rats, values for uptake of Fe^{59} reached a minimum of 5 to 8% at about 17 days after hypophysectomy. In the older rats, on the other hand, the lowest rate of Fe^{59} uptake, 3 to 5%, was reached at 8 days after removal of the pituitary (Fig. 1). A comparison of responsiveness to anemic plasma of the 2 age groups is presented in Table I.

In the assay of anemic plasma 3 days following hypophysectomy, in the younger rat the ratio of treated to control iron uptake was

TABLE I. Effect of Anemic Plasma on Uptake of Fe^{59} in 4-Wk-Old and 8-Wk-Old Rats at Intervals of 3, 10, 15, 16, and 90 Days after Hypophysectomy.*

Age at operation, wk	Substance	Unoperated control	Days post-hypophysectomy when Fe^{59} was inj. 3	10	15	16	90
4	Anemic plasma	45 †	35			16	24
	Control	38	30			8	15
8	Anemic plasma	44.6	17.0	18.1	18		
	Control	37.5	10.1	4.0	5		

* Each interval represents avg for 5 rats/group.
† These figures represent % incorporation of Fe^{59}.

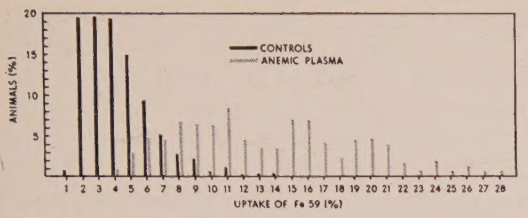

FIG. 2. Distribution of % Fe^{59} incorporation in control hypophysectomized rats and in those treated with anemic plasma.

1.2, while in animals used for assay 16 days after operation, the ratio was 2.0. As would be expected, the relative response to anemic plasma was greater at the time when rate of erythropoiesis was minimal. When the anemic plasma was assayed in older group of rats, the ratios at 3, 10, and 15 days were respectively 1.7, 4.5, and 3.6. These values indicate that in rats of this age, the maximum responsiveness to erythropoietin also occurred at the time of minimum erythropoietic activity, although this was several days earlier than in the case of the 4-week-old animals.

For assay purposes, we use rats that are hypophysectomized when they are 8 weeks old because the minimum rate of erythropoiesis is reached earlier and there is less variability in response than in rats that are subjected to surgery at an earlier age. B. *Variability of assay using hypophysectomized animals as recipients.* To give an indication of variability inherent in the assay procedure described in this and the previous paper(2), we have compiled values for uptake of Fe^{59} for each control hypophysectomized animal (8 weeks old) used in 45 previous experiments. "Control animals" consist of all rats injected with normal plasma or saline or that were uninjected. These controls did not differ significantly from one another. Fig. 2 indicates that 74% of control hypophysectomized animals had Fe^{59} uptake values of 5% or less. In making a similar compilation of the Fe^{59} uptake values for all animals that received anemic plasma (from rabbits or rats), we found that only 0.6% had an Fe^{59} uptake of less than 5% and that 70% had an Fe^{59} uptake exceeding 10%. Since Fig. 2 includes animals from 45 different experiments, many different lots of anemic plasma are represented. This probably accounts for the very broad spread in results.

C. *Polycythemic rats.* In rats with hematocrit values ranging between 70 and 75%, the rate of erythropoiesis, as determined by uptake of Fe^{59}, declined from 32 to 4%. After 8 days of red cell injection, when the rate of new red cell formation was at this minimum, anemic plasma was injected for 3 days. The ratio of treated to control iron uptake values was 6.0 as compared with a ratio of 1.4 for normal rats.

D. *Hyperoxic rats.* Rats subjected to an atmosphere of 85-95% O_2 displayed a diminished rate of erythrocyte production as evidenced by reduction in uptake of Fe^{59} by erythrocytes from control values of 32 to 8% at the 10-day interval. After administration of anemic plasma to these animals, the ratio of treated to control values was 3.0 (normal rats 1.4). Rats maintained in the atmosphere of high O_2 for more than 2 weeks did not survive.

E. *Fasted rats.* The decline in rate of erythropoiesis and increased responsiveness to anemic plasma in starved rats are illustrated in Fig. 3. When the animals lost about one-third of their body weight, their iron uptake values were at a minimum, while their response to injections of anemic plasma was high. The ratios of iron uptake values in treated over controls at 0, 3, 4, 6, and 7 days of fasting were respectively 1.4, 4.7, 5.4, 3.6,

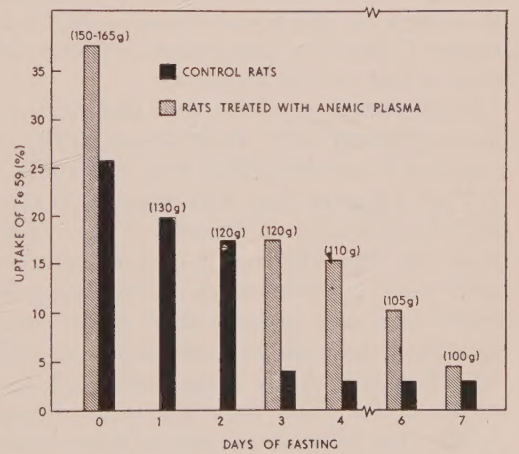

FIG. 3. Effect of duration of starvation on Fe^{59} incorporation and on response to erythropoietin in 8-wk-old rats.

TABLE II. Relative Sensitivity of Assay Preparations to Anemic Plasma in Terms of % Fe^{59} Incorporation.

Recipient*	Plasma inj. Anemic	Control
Polycythemic	24	4
Normal	44	32
High O_2	24	8
Normal	46.5	34
Starved (4 days prior to Fe^{59} admin.)	15	3
Normal	38	27
DNP to normal rat	42	36
Normal	43	22

* Each figure is avg of 5 individual values.

and 1.4. The sharply diminished response in the later stage of starvation probably reflects the over-all deterioration of the animals.

F. *Administration of dinitrophenol* to normal rats resulted in increased rate of erythropoiesis; about 70% greater than that in controls. Animals treated with dinitrophenol showed a response to anemic plasma about 12% greater than that to saline. Untreated animals had a response to anemic plasma that was aboult 100% greater than that to saline. A comparison of the responses to anemic plasma of these variously treated animals is given in Table II.

Discussion. In previous papers(2,3) we presented data indicating that erythropoiesis as measured by incorporation of Fe^{59} or reticulocyte values, declines following hypophysectomy. Administration of anemic plasma to animals in which erythropoiesis is at a minimum elicits an exaggerated response in terms of new red cell formation.

In the present paper we show that rats hypophysectomized at 4 weeks of age, undergo a decline in erythropoiesis that is considerably more gradual than that observed in rats hypophysectomized at 8 weeks of age. The reason for this difference is obscure but certainly deserves further study. The data presented here also indicate that as the new equilibrium between red cell formation and red cell mass is slowly being established, the uptake of Fe^{59} rises from low values found shortly after hypophysectomy to those about midway between pre-operative level and the lowest point. They remain at that level for at least 90 days, (length of our observations).

The following hypothesis was suggested previously in part as an explanation of our data(2,3). After hypophysectomy, the overall metabolic requirement of the animals drops rapidly to a level that is a fraction of that in the normal animal. There is, therefore, a decrease in the demand for oxygen by the tissues. Since the red cell mass does not decrease significantly within the first 2 or 3 weeks, there exists a relative plethora of erythrocytes, analogous to that in an animal with a transfusion-produced polycythemia. This discrepancy between demand of tissues for O_2 and the amount of O_2 available manifests itself, in some manner, by bringing about a reduction in production of erythropoietin, and as a consequence, erythropoiesis falls to a minimum within a week after hypophysectomy. At this time, administration of anemic plasma rich in erythropoietin would be expected to increase markedly the production of red cells.

Because of the radical initial reduction in erythropoiesis with continuing natural death of red cells, the red cell mass declines slowly. This gradual decrease in O_2-carrying capacity lessens the discrepancy between O_2 supply and demand.

As the plethoric state that was established initially slowly diminishes, the rate of erythropoiesis that had decreased to a minimal value begins to rise until it is able to maintain the red cell mass at a level compatible with the new rate of demand for O_2 by the tissues.

According to this working hypothesis, other experimental conditions that likewise produce a discrepancy between the demand for O_2 and availability of O_2 should also alter the rate of erythropoiesis.

In the experiments with polycythemic rats, the oxygen-carrying capacity of the blood is increased with, probably, no increase in the metabolic requirement for oxygen. This situation is therefore analogous to that in the rat shortly after hypophysectomy and also results in a decreased rate of erythropoiesis and increased responsiveness to anemic plasma.

When animals are subjected to an environ-

ment of 85-95% O_2, there are small but significant increases in the amount of oxygen that is carried(5,6). As in the case of hypophysectomized and polycythemic animals, this condition, by increasing the discrepancy between the supply and demand for O_2, decreases the rate of erythropoiesis and heightens responsiveness to erythropoietin.

Animals subjected to starvation have been shown to have decreased basal metabolic rates very shortly following the onset of the fast (7). In acute starvation, a marked decrease in the tissue demand for oxygen exists without appreciable change in the number of circulating erythrocytes. Thus, a relative plethora of red cells exists in these animals and, like the other preparations discussed above, a decrease in erythropoiesis occurs. Acute caloric deprivation may have an appreciable effect on the synthesis of the plasma factor(s) involved in erythropoiesis. This possibility needs clarification. On the other hand, it is known that protein-deprivation in rats reduces the incorporation of Fe^{59} into the red cells.§

While the situations discussed above all tended to increase the responsiveness of animals to erythropoietin, the rats that were treated with dinitrophenol would be expected to increase their metabolic requirement for O_2 without an immediate, compensating increase in supply of O_2. This is the reverse of the previous cases, and if the hypothesis stated above is valid, the rate of erythropoiesis should increase and the responsiveness to erythropoietin should decrease, as they do. Preliminary experiments with the naturally-occurring metabolic stimulant triiodothyronine gave similar results.

These investigations suggest that formation of erythropoietin and consequently the rate of erythropoiesis are regulated not by the absolute O_2 tension of the blood, but rather by the relationship between O_2 tension of the blood and the oxygen demand by the tissues.

Summary and conclusions. 1. We studied the erythropoietic response to anemic plasma of a variety of experimental conditions in the rat. Rats subjected to hypophysectomy, an atmosphere of high O_2, starvation, and transfusion-induced polycythemia have a decreased rate of erythropoiesis and an exaggerated response to the administration of anemic plasma. 2. Treatment with dinitrophenol increases the rate of erythropoiesis and decreases the response to anemic plasma. 3. These findings are in agreement with the hypothesis that the rate of erythropoiesis is determined by the amount of erythropoietin, the production of which is regulated by the relationship between O_2 supply and demand, not by either factor alone.

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